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Arachidonic acid enhances TPA-induced differentiation in human leukemia HL-60 cells via reactive oxygen species-dependent ERK activation

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ABSTRACT

The phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), is a potent stimulator of differentiation in human leukemia cells; however, the effects of arachidonic acid (AA) on TPA-induced differentiation are still unclear. In the present study, we investigated the contribution of AA to TPA-induced differentiation of human leukemia HL-60 cells. We found that treatment of HL-60 cells with TPA resulted in increases in cell attachment and nitroblue tetrazolium (NBT)-positive cells, which were significantly enhanced by the addition of AA. Stimulation of TPA-induced intracellular reactive oxygen species (ROS) production by AA was detected in HL-60 cells via a DCHF-DA analysis, and the addition of the antioxidant, *N*-acetyl-cysteine (NAC), was able to reduce TPA+AA-induced differentiation in accordance with suppression of intracellular peroxide elevation by TPA+AA. Furthermore, activation of extracellular-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) by TPA+AA was identified in HL-60 cells, and the ERK inhibitor, PD98059, but not the JNK inhibitor, SP600125, inhibited TPA+AA-induced NBT-positive cells. Suppression of TPA+AA-induced ERK protein phosphorylation by PD98059 and NAC was detected, and AA enhanced ERK protein phosphorylation by TPA was in HL-60 cells. AA clearly increased TPA-induced HL-60 cell differentiation, as evidenced by a marked increase in CD11b expression, which was inhibited by NAC and PD98059 addition. Eicosapentaenoic acid (EPA) as well as AA showed increased intracellular peroxide production and differentiation of HL-60 cells elicited by TPA. Evidence of AA potentiation of differentiation by TPA in human leukemia cells HL-60 via activation of ROS-dependent ERK protein phosphorylation was first demonstrated herein.

1. Introduction

Acute myeloid leukemia (AML) is characterized by a blockage of myeloid differentiation, and most current treatment strategies kill cancerous cells by chemotherapeutic agents with a number of severe toxic side-effects. Therefore, an alternative treatment of AML is to use agents to modify tumor growth by inducing terminal differentiation, such as all-trans retinoic acid and As_2O_3, as terminal differentiation inducers to treat acute promyelocytic leukemia [1,2]. Extensive efforts are being directed toward exploring agents with potent activity of increasing terminal differentiation of AML.

12-*O*-tetradecanoylphorbol-13-acetate (TPA) was reported to induce differentiation of myeloid precursors and myeloid leukemia cells [3,4]. TPA showed inhibitory effects on the growth of leukemia HL-60 and K562 cells, and on several other cells such as MCF-7 and NIH3T3 [5–7]. Additionally, both pro- and anti-apoptotic effects of TPA were extensively reported. TPA induces apoptosis in prostate cancer cells via the release of a death receptor ligand and activation of an apoptotic cascade, and reduces cell viability by reducing the activity of extracellular-regulated protein kinases (ERKs) in leukemia cells [8,9]. In contrast to apoptosis

Abbreviations: AA, Arachidonic acid; AML, Acute myeloid leukemia; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; DMSO, Dimethyl sulfoxide; ERK, Extracellular regulated kinase; LY, LY294002; NAC, *N*-acetyl-cysteine; NBT, Nitroblue tetrazolium; JNK, c-Jun N-terminal kinase; PARP, Poly(ADP-ribose) polymerase; PD, PD98059; ROS, Reactive oxygen species; SB, SB203580; EPA, Eicosapentaenoic acid; SDS, Sodium dodecylsulfate; SP, SP600125; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

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induction, TPA inhibits singlet oxygen-induced apoptosis and suppresses DNA fragmentation induced by polychlorinated biphenyls in human leukemia HL-60 cells [10,11] Our recent studies demonstrated that TPA possesses a protective effect against baicalein and prostaglandin-induced apoptosis in human leukemia cells [12,13]. Although differentiation induction by TPA in leukemia cells was reported, studying the interaction of TPA with other chemicals to improve its differentiation effect is an important issue for clinically treating leukemia patients.

Arachidonic acid (AA) is a fatty acid with potent physiological activity through the production of active metabolites such as prostanoids, leukotrienes, and lipoxins [14,15]. Induction of apoptosis by AA in leukemia cells was reported. Arita et al. [16] indicated that AA-induced apoptosis of HL-60 cells was mediated by activation of a caspase cascade through cytochrome *c* release. Jung et al. [17] reported that AA inhibited the proliferation of HL-60 cells via inducing apoptosis through blocking DNA binding of the Myc87/Max85 dimer. Additionally, monocytes and macrophages, but not premonocytic cells, with the ability to actively metabolize AA, indicated that AA metabolism might be involved in the differentiation process of these cells [18,19]. Gonchar et al. [20] indicated no connection between AA release and prostanoid synthesis upon differentiation of leukemia U937 cells. Therefore, the role of AA in differentiation of leukemia cells is still unclear.

The human promyelocytic leukemia HL-60 cell line has been widely used to study the regulatory mechanism of hematopoietic cell differentiation. Collins [21] indicated that HL-60 cells differentiate into a monocytic/macrophage-like lineage by TPA. The aim of this study was to investigate the effects of AA on TPA-induced differentiation of human HL-60 leukemia cells. It is demonstrated that AA may enhance differentiation in TPA-treated HL-60 cells via reactive oxygen species (ROS)-dependent ERK activation.

2. Materials and methods

2.1. Cells

HL-60 is a poorly differentiated promyelocytic cell line and was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI 1640 supplemented with antibiotics (100 U/ml penicillin A and 100 U/ml streptomycin) and 10% heat-inactivated fetal bovine serum (FBS; Gibco/BRL, Grand Island, NY) and maintained in a 37 °C humidified incubator containing 5% CO_2. In the presence of AA, EPA, or TPA treatment, HL-60 cells were incubated at 400 μl of fresh RPMI containing 10% FBS, and indicated concentrations of AA, EPA, TPA were added for an addition 24 h.

2.2. Agents

The chemical reagents of TPA, AA, EPA, *N*-acetyl cysteine (NAC), PD98059, SB203580, SP600125, LY294002, and nitroblue tetrazolium (NBT) were obtained from Sigma Chemical (St. Louis, MO, USA). Antibody of α-tubulin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies of total and phosphorylated ERK, c-Jun N-terminal kinase (JNK), p38, and AKT were obtained from Cell Signaling Technology (Danvers, MA, USA). EPA and AA at the stock solutions of 100 mM were prepared by dissolving fatty acids in DMSO. Because both are very sensitive to oxidation, stock solutions were kept at −20 °C, under N_2 and in the dark. Immediately before use, EPA and AA stock solutions were diluted in a culture medium to reach the final concentration of 10 μM. The final concentration of DMSO in the culture medium did not exceed 0.5% and was not toxic to HL-60 cells.

2.3. Differentiation and adhesion assay

The differentiation and adhesion assay were carried out with 4×10^5 cells/well in 24-well plates, which were treated with TPA, AA, and TPA + AA for 24 h (TPA and AA were dissolved in DMSO, at a final concentration in media $< 0.02\%$). Control cells were treated with a similar concentration of DMSO. Numbers of TPA-differentiated cells were determined using an adhesion assay. Briefly, cells were fixed and incubated at 37 °C for 30 min after adding 0.5% crystal violet (in 20% methanol) and subsequently washed. After washing, the stain was eluted with 0.1 M sodium citrate (pH 4.2, in 50% ethanol), attached cells were observed microscopically and the intensity of crystal violet was measured at 600 nm in an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, MA, USA).

2.4. Western blotting

Total cellular extracts were prepared, and separated on 10% sodium dodecylsulfate (SDS)-polyacrylamide minigels, and transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Billerica, MA, USA). Membranes were incubated with 1% bovine serum albumin (BSA) and then incubated with specific antibodies overnight at 4 °C. Expression of protein was detected by staining with NBT and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma).

2.5. Detection of HL-60 cell differentiation by NBT reduction

HL-60 cells under different treatments were assayed by the NBT test. The assay was performed on cells dispersed in a final volume of 1 ml in the presence of NBT at a final concentration of 0.5 mg/ml. After 30 min of incubation at 37 °C, reactions were stopped by putting samples on ice. Then, the supernatant was removed, and crystals were dissolved in DMSO. The absorbance was read at 570 nm with an ELISA analyzer (Dynatech MR-7000; Dynatech Laboratories, Inc., Chantilly, VA, USA).

2.6. Measurement of ROS generation by intact cells

Intracellular production of ROS by HL-60 leukemia cells under different treatments was measured by oxidation of DCFH-DA to DCF. DCFH-DA is a non-polar compound that readily diffuses into cells, where it is hydrolyzed to the non-fluorescent polar derivative, DCFH, and thereby trapped within cells. If DCFH-DA is oxidized, it turns into the highly fluorescent DCF. HL-60 cells were incubated in the dark for 10 min at 37 °C with 100 nM DCFH-DA, then harvested, and resuspended in plain medium. 10^4 cells were acquired per sample, the fluorescence in HL-60 cells was analyzed using a FACScan (Becton Dickinson, Sunnyvale, CA) flow cytometer with excitation at 488 nm and emission at 530 nm.

2.7. Detection of differentiation by FACS analysis of CD11b expression

Cell surface expression of antigen CD11b was determined by fluorescence-activated cell sorting analysis. HL-60 cells under different treatments were harvested and washed once with phosphate buffered saline containing 0.5% bovine serum albumin. Aliquots of cell suspension were stained with CD11b-FITC conjugated antibody (Beckman Coulter, Inc., France) and analyzed with a Becton Dickinson flow cytometer (Franklin Lakes, NJ, USA).