Ursolic acid induces HL60 monocytic differentiation and upregulates C/EBPβ expression by ERK pathway activation

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Ursolic acid (UA), a pentacyclic triterpenoid compound, is widely distributed in the plant kingdom and has a broad range of biological effects. This study was carried out for the first time to investigate the potential role of UA in the differentiation of human leukemia HL60 cells and the underlying mechanisms in it. UA could induce differentiation of HL60 cells into the monocytic lineage, as assessed by the morphological change, nitroblue tetrazolium reduction assay, and expression of CD14 and CD11b surface antigens. Moreover, UA activated the extracellular signal-regulated kinase (ERK) pathway in both dose-dependent and time-dependent manners. Inhibiting ERK pathway activation with PD98059 could significantly block the differentiation induced by UA. Consistent with the induced differentiation, the upregulation of CCAAT/enhancer-binding protein β by UA was also eliminated by PD98059. Taken together, the results reported here show that UA can promote the monocytic differentiation of HL60 cells and increase the expression of CCAAT/enhancer-binding protein β by activating the ERK pathway, suggesting that UA could be a potential candidate as a differentiation-inducing agent for the therapeutic treatment of leukemia. *Anti-Cancer Drugs* 22:158–165 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Acute myeloid leukemia (AML) is a hematologic malignancy characterized by a block in differentiation that leads to the accumulation of immature cells. Induction of differentiation is therefore becoming the ideal therapeutic strategy in a subset of AML [1]. Substances such as 1,25-dihydroxyvitamin D3 (DHD3) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) inhibit proliferation and induce differentiation of AML HL60 cells into the monocyte/macrophage lineage [2], whereas all-trans-retinoic acid (ATRA) causes differentiation into morphologically and functionally mature granulocytes [3].

Ursolic acid (UA), a pentacyclic triterpene acid, is widely distributed in food, medicinal herbs, and other plants. Earlier studies have shown that UA can inhibit proliferation and induce apoptosis in many kinds of cancer cell lines, including leukemia, prostate, lung, endometrial, and melanoma cancer cells [4–9]. Moreover, it has been shown that this triterpenoid acts at different stages of tumor development, including inhibition of tumorigenesis, metastasis, and angiogenesis, as well as induction of cell differentiation and apoptosis. Lee *et al.* [10] found that UA acted as a differentiation agent to induce morphological change of F9 teratocarcinoma stem cells into endoderm cells. Recently, Bonaccorsi *et al.* [11] reported that UA treatment promotes differentiation of human tumor cell lines from melanoma, glioblastoma, and thyroid anaplastic carcinoma, which involves the inhibition of the endogenous reverse transcriptase (RT) activity. However, the potential effect of UA on the differentiation of leukemia cells has not yet been examined.

In this study, we investigated the differentiating effect of UA on HL60 cells for the first time. Our data show that UA induces monocytic differentiation through activation of the extracellular signal-regulated kinase (ERK) signaling pathway. Moreover, we proved that such differentiation involves the upregulation of CCAAT/enhancer-binding protein β (C/EBPβ), which has been reported as an important transcription factor for monocytic differentiation.

Materials and methods

Reagents

UA (purity > 98%) was dissolved in dimethyl sulfoxide (DMSO; Sigma, USA) and stored at −20°C. Nitroblue tetrazolium (NBT), TPA, and PD98059 were bought from Sigma (Sigma, St Louis, Missouri, USA). Goat antiphospho-ERK1/2,rabbit anti-ERK1/2, mouse antiphospho-p38 mitogen-activated protein kinases (MAPK), rabbit anti-p38 MAPK, rabbit antiactive c-Jun NH2-terminal kinase 1/2 (JNK1/2), and mouse anti-JNK1/2 were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, California,

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USA). Rabbit anti-C/EBPβ and rabbit anti-c-myc were obtained from Bioworld Technology Inc. (Minneapolis, Minnesota, USA).

Cell culture

The human leukemia cells used were HL60, U-937, and THP-1 (ATCC Manassas, Virginia, USA), all of which were cultured in RPMI 1640 medium (Gibco/BRL, Grand Island, New York, USA) supplemented with 10% fetal bovine serum. The cells were subcultured two to three times weekly to maintain a log-phase growth. To test proliferation, the cells were seeded at 5×10^4/ml in 24-well plates and allowed to grow for 5 days in the growth medium containing various amounts of UA or 0.02% (v/v) DMSO as vehicle control. UA dissolved in DMSO was added to the medium in a serial dilution process (the final DMSO concentration in all the assays did not exceed 0.02%). Cell numbers were counted using a Burker chamber (two countings/sample).

Differentiation assay

(i) NBT reduction assay: the NBT reducing activity was determined by the method described by Poon *et al.* [12]. In brief, after treatment, 1×10^6 cells were harvested, incubated with 2 mg/ml NBT and 1 mg/ml TPA in a serum-free medium for 30 min at 37°C, then washed with 70% methanol, and finally lysed using 500 µl of 2mol/l KOH overnight. The nitroblue diformazan deposit was dissolved in 600 µl/sample of DMSO and absorbance was read at 570 nm. (ii) Analysis of morphological changes: the cells were collected on the slides, fixed in methanol and stained with the Wright–Giemsa staining solution, rinsed in deionized water, air-dried, and then observed under a light microscope (YS100; Nikon, Japan). (iii) Flow cytometric analysis: A direct immunofluorescence staining technique was used to detect the cell surface markers. The cells were washed twice with the wash buffer (phosphate buffered saline, 0.1% sodium azide, and 0.2% (w/v) bovine serum albumin), and then incubated on ice with the anti-CD11b antibody conjugated with phycoerythrin and the anti-CD14 antibody conjugated with fluorescein isothiocyanate for 30 min or with a particular isotype control (ebioscience, San Diego, California, USA). The cells were then washed and resuspended in 500 µl of wash buffer, and finally analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA).

RNA extraction and semiquantitative reverse-transcription PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, California, USA). RT-PCR was performed with Moloney murine leukemia virus (Promega, Madison, Wisconsin, USA) following standard protocols. For PCR, The primer sequences were c-myc: 5′-CTGAGGTGGTT CATACTGAGCAAG-3′, and 5′-ACGCTGACCAAGGTG TTGGTAG-3′; C/EBPβ: 5′-CCACGGCCACGGACACC TTCG-3′, and 5′-GCGCGGCCTCCCTGCTCTGA-3′ [13]; glyceraldehyde 3 phosphate dehydrogenase : 5′-AAGGTC GGAGTCAACGGATT-3′, and 5′-CTGGAAGATGGTGA TGGGATT-3′ [14]. The amplification procedure was at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, at 57°C for 45 s, at 72°C for 45 s, and finally at 72°C for 10 min. Aliquots of PCR products were electrophoresed on 1.2% agarose gels and PCR fragments were visualized by ethidium bromide staining. The DNA bands were analyzed using the Quantity One software (Bio Rad, Hercules, California, USA).

Western blotting

After various treatments for indicated intervals, the cells were lysed in a lysis buffer. The cells used for phospholysates were lysed with a lysis buffer enriched with protease inhibitors cocktail, 1 mmol/l sodium fluoride, and 1 mmol/l sodium orthovanadate [15]. Proteins (40 µg) were separated on a 12% SDS-polyacrylamide gel and transferred electrophoretically onto polyvinylidene difluoride membranes (Millipore, Bedford, Massachusetts, USA). The membranes were blocked with 10% milk in Tris–buffered saline/0.1% Tween 20 for 2 h, and subsequently blotted with primary antibodies and then with a horseradish peroxidase-conjugated secondary antibody for 1 h. The protein bands were visualized with an enhanced chemiluminescence detection system (Amersham, UK). Protein levels were quantified by density analysis using the Quantity One software (Bio Rad).

Results

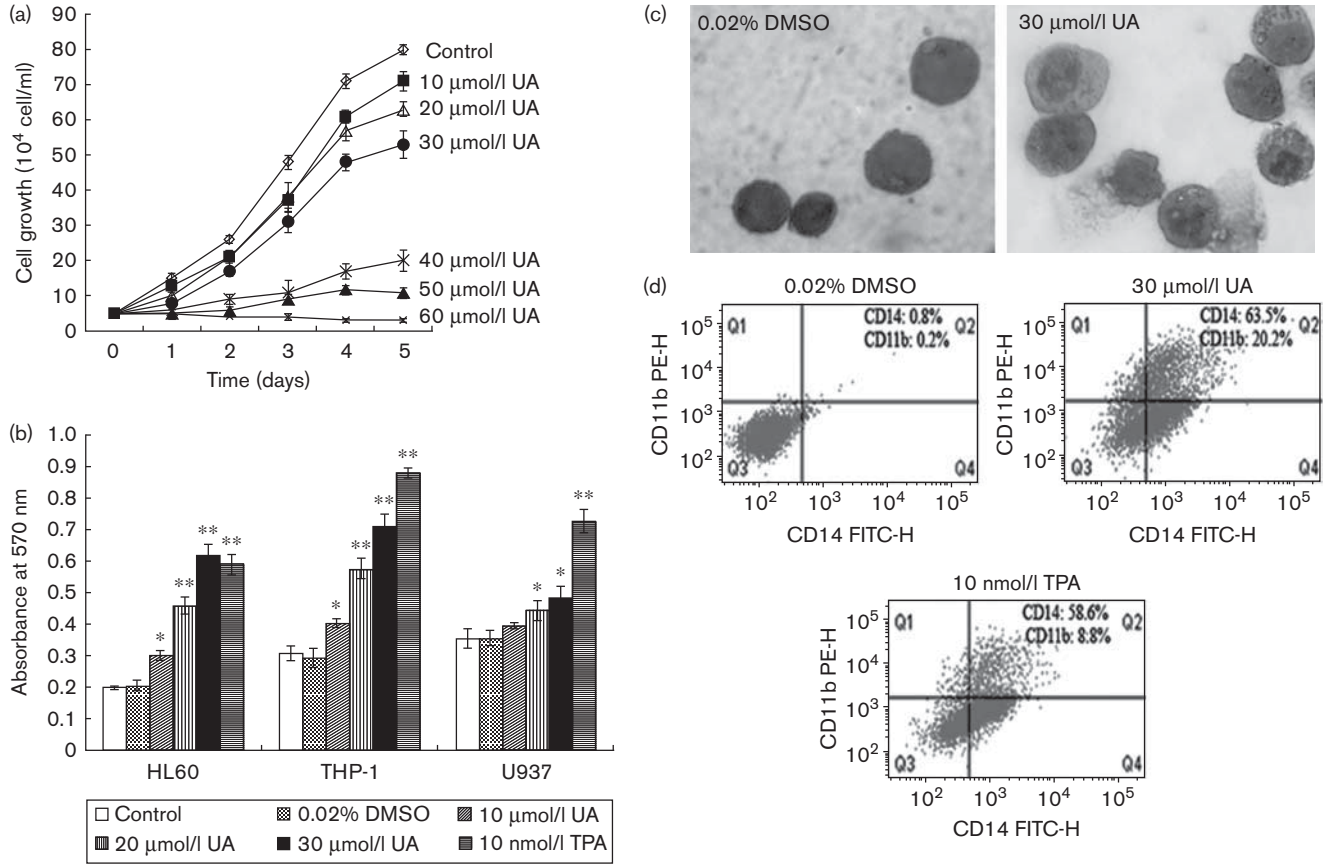
Effects of ursolic acid on the growth of HL60 cells

To test the effect of UA on the growth of HL60 cells, they were cultured in the absence and presence of UA at various concentrations (10–60 µmol/l). The results in Fig. 1a showed that the efficiency of cell growth inhibition increased dramatically as the concentration of UA increased. A linear regression of the data in Fig. 1a allowed the prediction of the IC_{50} (32.6 ± 2.4 µmol/l with 5-day treatment) of UA for HL60 cells. It was noted that the efficiency of growth inhibition was also correlated with exposure time at a given UA concentration. However, the dependence on exposure time was only significant when the UA concentration was below IC_{50}. When UA was applied at higher concentrations, the effect of contact time became less significant. Although the inhibitory effects were apparent at concentrations of 10, 20, and 30 µmol/l of UA, no cytocidal effects were observed under these conditions. Thus, these concentrations were used for subsequent studies.

Ursolic acid induces HL60, THP-1, and U937 leukemia cell lines differentiation

UA induced differentiation of HL60 myeloid leukemia cells, as shown by morphology, NBT reduction, and immunophenotyping. The TPA-stimulated formation of superoxide is a typical characteristic of mature myeloid cells [16]. The NBT reduction assay was performed to show mature

Fig. 1



Effects of ursolic acid (UA) on the growth and differentiation of HL60 cells. (a) UA affects the growth of HL60 cells. Values are means ± SD of three parallel experiments. (b) UA induces nitroblue tetrazolium (NBT) reduction activity. HL60, THP-1, and U937 cells were treated with 10, 20, and 30 μmol/l of UA, 0.02% of dimethyl sulfoxide (DMSO, vehicle control), and 12-*O*-tetradecanoylphorbol 13-acetate (TPA, positive control) for 5 days and then the NBT reduction assay was performed. Significant differences between the control and the treated group, analyzed by Dunnett's test are indicated by asterisks: $*P<0.05$ and $**P<0.01$, $n=3$. (c) UA induces morphologic changes consistent with monocytic differentiation. After treatment for 5 days with 30 μmol/l of UA, cytospin preparations were prepared and the cells were stained with Wright–Giemsa stain (magnification 400). (d) UA induces immunophenotypic changes consistent with monocytic differentiation induced by TPA. After the treatment for 5 days, HL60 cells were stained with CD11b-phycoerythrin (PE) and CD14-fluorescein isothiocyanate (FITC), and flow cytometric analysis was performed. CD14 FITC-H, CD14 FITC-Height; CD11b PE-H, CD11b-Height.

differentiation induced by different concentrations of UA. After treatment for 5 days, a significant increase in NBT reduction was observed in a dose-dependent manner (Fig. 1b). We evaluated the effect of differentiation of UA on two additional leukemia cell lines, which are THP-1 and U937, using the NBT reduction assay. The results show that UA can induce differentiation of all the three cells; however, HL60 and THP-1 cells are more sensitive than U937 (Fig. 1b). Interestingly, after treatment with UA for 3 days, THP-1 became adherent and developed a macrophage-like appearance, whereas such effects were not significant in HL60 and U937 cells (data not shown).

Using Wright–Giemsa staining, undifferentiated control HL60 cells were found to be predominantly myelocytes with round, regular cell margins, and large nuclei. Treatment with UA (30 μmol/l) resulted in condensed nuclei, and abundant cytoplasm and vacuoles, suggesting the monocytic differentiation of HL60 cells (Fig. 1c). Immunophenotyping with CD14, a marker of monocytic differentiation, and CD11b, which is upregulated during myelomonocytic differentiation [17,18], further confirmed the differentiation-inducing effect of UA. Compared with vehicle control, the number of CD14-positive cells increased up to 63.5% after the treatment with 30 μmol/l of UA for 5 days, whereas the CD11b marker was expressed in 20.2% of the cells, implying that UA could induce HL60 monocytic differentiation (Fig. 1d).

Effect of ursolic acid on the expression of C/EBPβ and c-myc

Transcription factor C/EBPβ plays an important role in DHD3-induced monocytic differentiation [19]. The regulation of c-myc expression is also reported to be a potential mechanism of monocyte/macrophage-like terminal

differentiation [20]. To further examine whether UA would induce myeloid differentiation, C/EBPβ and c-myc were examined by semiquantitative RT-PCR and western blotting. Consistent with monocytic differentiation, after treatment with 30 μmol/l of UA for 5 days, the mRNA and protein levels of C/EBPβ were upregulated by 1.8-fold and 2.0-fold, respectively. Meanwhile, the expression of c-myc was downregulated to an undetectable level at 30 μmol/l (Fig. 2).

Ursolic acid triggers ERK1/2 phosphorylation

Some differentiation inducers can activate MAPK in HL60 cells, which is required to elicit differentiation and growth arrest [21,22]. To investigate whether the MAPKs pathway is involved in the UA-induced differentiation, we first examined the phosphorylation of ERK, JNK, and p38 by western blotting within 1 h after the incubation of UA. Our data showed that UA activated the ERK pathway in a dose-dependent manner, but had no effect on JNK and p38 phosphorylation (Fig. 3a and b). In a time-course study, the ratio of phosphor-ERK1/2 to total ERK1/2 was gradually increased by UA from 15 min to 1 h (Fig. 3c and d).

Ursolic acid induces HL60 differentiation and upregulates C/EBPβ by ERK pathway activation

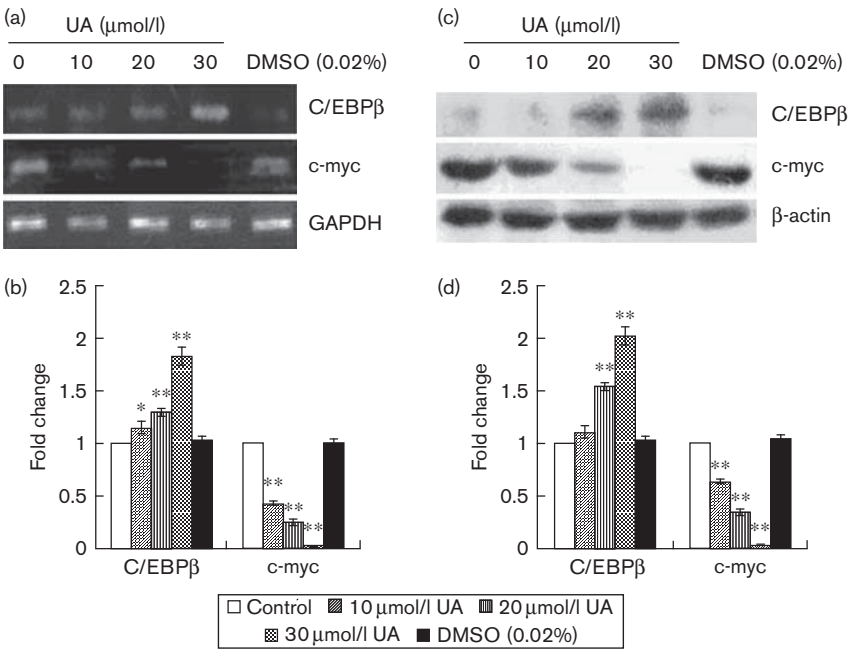
To further determine whether the activation of ERK signaling is responsible for HL60 cell differentiation induced by UA, we used PD98059 to study the expression of cell surface differentiation markers. PD98059 is a specific inhibitor to mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) and selectively inhibits ERK activation. When the HL60 cells were pretreated with PD98059 (30 μmol/l) for 1 h before the addition of UA (30 μmol/l) for further incubation of 5 days, it could completely block the expression of CD14 and CD11b (Fig. 4a). This suggested that UA induced differentiation in HL60 cells through ERK activation. Moreover, the upregulation of C/EBPβ by UA was also inhibited by PD98059 (Fig. 4b and c). These results indicated that the induction of the C/EBPβ gene expression might be involved in the UA-induced monocytic differentiation of HL60 cells.

Discussion

Differentiation therapy in acute promyelocytic leukemia with ATRA has made great breakthroughs since 1986. However, there are still several drawbacks, such as the fetal 'ATRA syndrome' and the development of resistance to this drug [23,24]. Therefore, increasing efforts have been focused on developing novel and effective differentiation inducers with less adverse effects in the recent years.

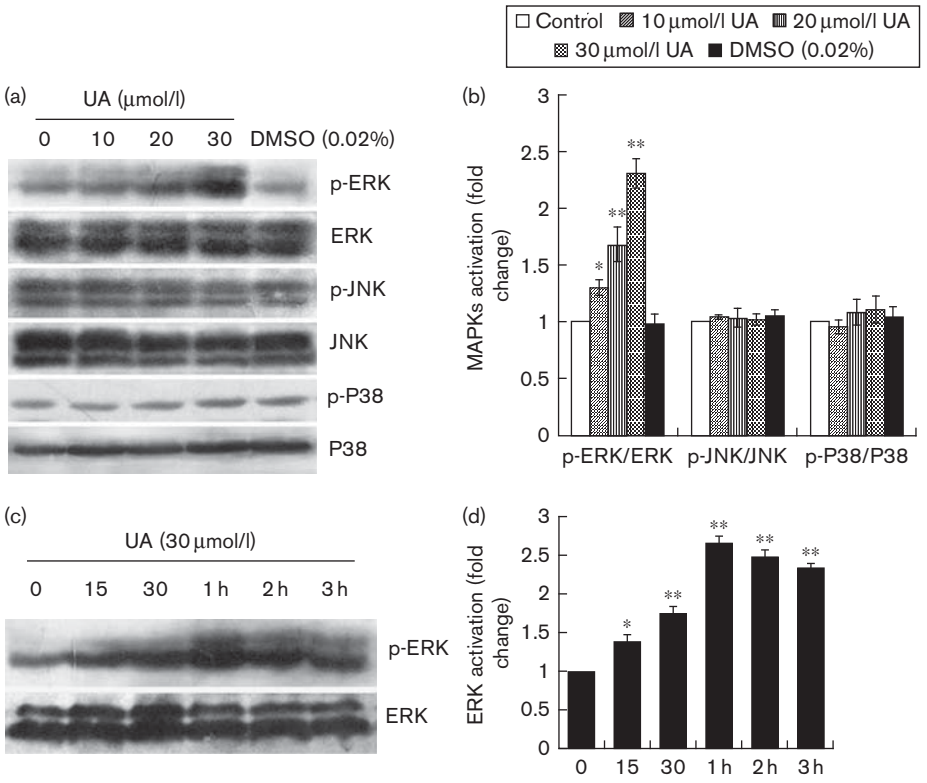
To the best of our knowledge, this is the first study to show that UA is an effective inducer of monocytic differentiation of HL60 cells. This effect of UA was confirmed with morphological analysis using Wright–Giemsa

Fig. 2



Ursolic acid affects the mRNA and protein expression of CCAAT/enhancer-binding protein β (C/EBPβ) and c-myc in HL60 cells. (a and c) mRNA and protein expression of C/EBPβ and c-myc during the UA-induced differentiation of HL60 was examined by RT-PCR. (b and d) Quantitative results of these experiments. Density value of each band was normalized to β-actin control and expressed as fold change compared with the control. Error bars indicate the range of the determinations. Significant differences between the control and the UA-treated group, analyzed by Dunnett's test are indicated by asterisks: $*P<0.05$ and $**P<0.01$, $n=3$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Fig. 3

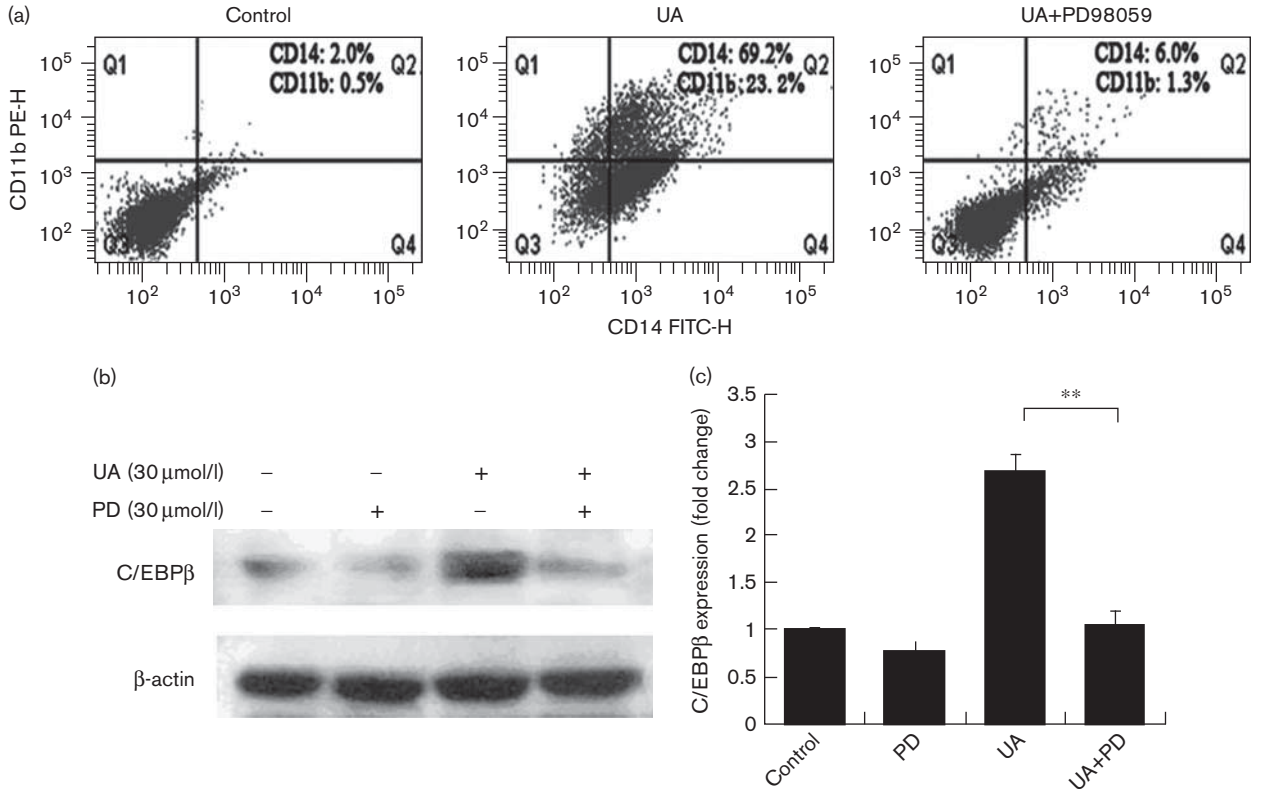


Effects of ursolic acid (UA) on the activation of mitogen-activated protein kinases (MAPKs) pathway in HL60 cells. (a) Phosphorylation and total extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and P38 in HL60 cells were respectively examined by western blotting within 1 h after the treatment with different concentrations of UA and 0.02% of dimethyl sulfoxide (DMSO). (c) Phosphorylation and total ERK in HL60 cells were examined after treatment with 30 μmol/l of UA at the indicated the timepoints (0, 15, 30 min, 1, 2, 3 h). (b and d) Quantitative results of these experiments. Error bars indicate the range of the determinations. Significant differences between the control and the UA-treated group, analyzed by Dunnett's test are indicated by asterisks: $*P<0.05$ and $**P<0.01$, $n=3$.

staining, NBT reduction test, and expression of cell surface differentiation markers (CD11b and CD14). After 5 days of treatment by 30 μmol/l of UA, the specific monocytic surface marker CD14 was increased significantly. Together with the change in morphology and the increase in NBT reduction capability, this suggested that UA induces HL60 cells to differentiate mainly toward the monocytic lineage. In addition, we also investigated the growth inhibition effect of UA against HL60 cells. It is worth mentioning that UA treatment did not change the cell cycle distribution of HL60, as shown by our flow cytometry measurement (data not shown), indicating that the antiproliferation and differentiating effect is not directly related to cell cycle arrest. As the positive control here, TPA can efficiently induce terminal differentiation of HL60 cells into a monocyte/macrophage-like phenotype [25]. According to our data, the differentiating effect of TPA at 10 nmol/l is equivalent to that of UA at a concentration of 30 μmol/l, which suggests that TPA is much more potent than UA (Fig. 1c). However, the high toxicity of TPA restricts its clinical application. In contrast, accumulating evidences about the safety study have shown that UA causes very little harm to normal cells, such as the serum-free mouse embryo cell line and the primarily cultured normal mouse hepatocytes [26,27].

Many studies have suggested that the MAPKs signaling pathway plays an important role in the monocytic and granulocytic differentiation of different leukemic cells induced by various differentiating agents, including DHD3, TPA, and ATRA [28,29]. However, earlier research on the effect of UA on MAPKs has shown mixed results. Achiwa *et al.* [30] reported that UA (50 μmol/ml) inhibited the ERK pathway in endometrial cell lines, whereas Ikeda *et al.* [15] showed that a lower dose of UA (4 μmol/l) induced the activation of ERK1/2 within 30 min. Our data show that the UA-induced HL60 cell differentiation is mediated by the activation of the ERK pathway, as indicated by the increased levels of phosphor-ERK1/2 in a concentration-dependant and time-dependant manners. In support of this observation, we also found that the UA-induced cell differentiation was blocked by PD98059, a specific MEK inhibitor. The preincubation of cells with 30 μmol/l of PD98059 1 h before the addition of 30 μmol/l of UA significantly inhibited the expression of differentiation markers, suggesting that ERK activation is

Fig. 4



Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) inhibitor PD98059 blocks ursolic acid (UA)-induced expression of the differentiation marker and CCAAT/enhancer-binding protein β (C/EBPβ). (a) After incubating for 5 days in the presence of 30 μmol/l of UA and a combination of 30 μmol/l of UA and 30 μmol/l of PD98059, the cells were harvested, stained with CD11b-phycoerythrin (PE) and CD14-fluorescein isothiocyanate (FITC), and analyzed using the two-parameter FACSCalibur flow cytometry. (b) After incubating for 5 days in the presence of 30 μmol/l of UA, 30 μmol/l of PD98059, or a combination of 30 μmol/l of UA and 30 μmol/l of PD98059, cell lysates were prepared and subjected to western blotting with the antibody against C/EBPβ. Density value of each band was normalized to β-actin control and expressed as fold change compared with the control. (c) Highly significant differences between the combined treatment group and the UA-treated group, analyzed by Dunnett's test are indicated by asterisks: $^{**}P<0.01$, $n=3$.

intimately involved in the UA-induced differentiation. Compared with UA, the differentiating effect of TPA on HL60 is also mainly because of the activation of ERK1/2 [28], but is still regulated by other pathways such as phosphatidylinositol 3-kinase and Smad [31,32], and requires the action of two oncogenes, c-fes and c-fms [33,34]. The c-fms gene that encodes for the macrophage colony-stimulating factor receptor, which is a tyrosine kinase growth factor receptor essential for macrophage development, is regulated by the coordinated effects of three transcription factors, PU.1, AML1, and CEBP [35,36]. In addition, TPA-induced differentiation of HL60 is associated with the cell cycle arrest at the G1 phase and the induction of cyclinD1, $p21^{waf1}$, and $p27^{kip1}$ [28,37], whereas no significant cell cycle arrest happened in the UA-treated HL60 cells.

C/EBPβ is an important transcription factor for monocytic differentiation. It binds to retinoblastoma protein forming a complex, and acts to regulate the monocytic differentiation marker CD14 [38]. When AML cells were treated with 1, 25-dihydroxyvitamin D3, the expression of C/EBPβ protein was paralleled by the induction of monocytic differentiation markers [39]. Here, we showed by RT-PCR and western blot analysis that UA upregulated C/EBPβ through ERK pathway activation during monocytic differentiation. Furthermore, we also found that the differentiation of HL60 cells and the upregulation of C/EBPβ by UA was accompanied by a decrease of c-myc, which has been proved to be a negative regulator of terminal differentiation of HL60 [20]. Further studies are necessary to determine whether the UA-induced differentiation of HL60 cells depends on the regulation of these transcription factors.

Other triterpenoid compounds, such as CDDO [2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid] and CDDO-imidazolide (CDDO-Im), have been shown to induce cell differentiation in myeloid leukemia cells. Even though these triterpenoid compounds have a similar chemical structure, the effect and mechanism of differentiation are different from each other. CDDO potently induces

granulocytic differentiation in several leukemic cell lines and patient-derived primary AML blasts by translationally enhancing the expression and function of C/EBPα [40], whereas its derivative (CDDO-Im) and UA have been proved to induce monocytic differentiation. Increased C/EBPβ expression is also critical for CDDO-Im-induced differentiation, which is partially dependent on ERK activation and TGF-β-mediated Smad activation [38].

In summary, this is the first study to provide evidence that UA induces monocytic differentiation of HL60 cells and the expression of C/EBPβ through the activation of the ERK signaling pathway. Further understanding of the mechanisms of UA-induced differentiation could allow the identification of the key molecular target for this compound to facilitate the development of new pharmacological agents with potential therapeutic value and fewer side effects for the treatment of leukemia.

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