Extracellular Signal-regulated Kinase (ERK) is Required for Water Extract of *Nardostachys chinensis*-Induced Differentiation in HL-60 Cells

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The root and rhizomes of *Nardostachys chinensis* belonging to the family Valerianaceae has been used for medicinal therapy in Korean traditional medicine. The parts have been especially used to elicit stomachic and sedative effects. Our previous studies reported that the water extract of *N. chinensis* has induced granulocytic differentiation in human promyelocytic leukemia (HL-60) cells. The Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases involved in the regulation of various cellular responses, such as cell proliferation, differentiation and apoptosis. In this study, we investigated the signaling pathways on the HL-60 cell differentiation induced by *N. chinensis*. Activation of extracellular signal-regulated kinase (ERK) increased time-dependently in differentiation of HL-60 cells induced by *N. chinensis*. Activation of p38 increased slightly at 24 h after *N. chinensis* treatment, but activation of c-jun N-terminal kinase (JNK) was unaffected. Inhibitor of ERK (PD98059) significantly reduced NBT reduction activity induced by *N. chinensis* in HL-60 cells. In contrast, p38 inhibitor (SB203580) did not inhibit the cell differentiation. These results indicated that activaiton of ERK may be involved in HL-60 cell differentiation induced by *N. chinensis*.

Key words : *Nardostachys chinensis*, HL-60 cells, Differentiation, MAPKs

Introduction

The roots and rhizomes of *Nardostachys chinensis (N. chinensis)* belonging to the family Valerianaceae has been in used traditional medicine to elicit stomachic, anti-arrhythmic effect and sedative effect[1]. The plants is known to be rich in sesquiterpenoids[2], which have been found to exhibit antimalarial, antinociceptive[3], and cytototic activities[4], as well as to enhance nerve growth factors[5]. In the previous study, we demonstrated that *N. chinensis* potently inhibits the proliferation of human promyelocytic HL-60 cells via the G1 phase cell cycle arrest and granulocytic differentiation induction[6].

Human promyelocytic leukemia HL-60 cells are differentiated into macrophage/monocytic lineage[7,8] or granulocytic lineage[9]. The differentiation of HL-60 cells activates a variety of protein kinases including protein kinase C and mitogen-activated protein kinase[10-13]. Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases involved in the regulation of various cellular responses, such as cell proliferation, differentiation and apoptosis[14,15]. Based on structural differences, they are classified into three subfamilies: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK/SAPK) and p38 kinase. The ERK pathway is mainly activated by mitogens and growth factors, and plays a major role in the regulation of cell growth, survival and differentiaton[16,17]. In contrast, the JNK and p38 pathways are activated in response to chemicals and environmental stress and the activation is frequently associated with an induction of apoptosis[17,18]. MAPKs signaling pathway is involved in the monocytic and granulocytic differentiation of leukemia cell lines. The ERK pathway is required for the differentiation of leukemia cells into monocytes and granulocytes[19-21], but Activation of the JNK pathway is associated with monocytic differentiation[22,23], while the p38 pathway has a negative regulatory effect on monocytic differentiation[23].

In this study, we investigated MAPKs signaling pathways

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on the human promyelocytic leukemia HL-60 cell differentiation induced by *N. chinensis*.

Materials and Methods

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), nitrotetrazolium blue chloride (NBT), phorbol 12-myristate 13-acetate (PMA), protease inhibitor cocktail, and anti-β-actin were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). 2'-Amino-3'-methoxyflavone (PD98059) and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580) were purchased from Calbiochem (California, USA). RPE-conjugated anti-CD11b and FITC-conjugated anti-CD14 antibodies were purchased from DAKO (Glostrup, Denmark). Anti-ERK, anti-phospho-ERK, anti-p38, anti-phospho-p38, anti-JNK, and anti-phospho-JNK antibodies were purchased from Cell Signaling Technology INC. (Beverly, MA, USA).

1. Preparation of *Nardostachys chinensis* extracts

The roots and rhizomes of *Nardostachys chinensis* were extracted with distilled water (100g/1L) at 100℃ for 3 h. The extract was centrifuged at 2000 rpm for 15 min to remove the insoluble ingredients. The supernatant was then filtered through Whatman no. 4 filter paper in a Buchner funnel under vacuum. The filtrate was freezing dried after stored at -20℃ for overnight. The yield (W/W) of the extract was about 12.82%. Lyophilized extract was dissolved in PBS at a concentration 40 ㎎/㎖ and stored at -20℃ and diluted in cell culture medium before use.

2. Cell culture

HL-60 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and routinely cultured in RPMI 1640 medium (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Burlington, ON, Canada), 100 U/㎖ penicillin, 100 ㎍/㎖ streptomycin at 37℃ in humidified 95% air/5% CO_2 incubator.

3. Determination of cell viability

Exponentially growing cells were seeded into a 24 well plate at 1×10^5 cells/well in duplicate. The cells were treated with increasing concentrations of N. Rhizoma for 24 h. After the indicated time periods, 100 ㎕ of 5 ㎎/㎖ MTT was added to each wells and incubated for 4 h. Water-insoluble MTT-formazan crystals were solubilized by adding equal volume of solubilization solution (10% SDS/0.01 N HCl) and incubating the plate overnight in humidified atmosphere of 5% CO_2 at 37℃. The amount of formazan was determined at 570 ㎚ using SpectraMAX 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Percentage of the cell viability was calculated using the equation: (mean OD of treated cells/mean OD of control cells) × 100.

4. Differentiation assay

1) NBT reduction assay

HL-60 cells (1×10^6 cells/60 ㎜ dish) were cultured with N. chinensis in RPMI-1640 medium containing 10% FBS for 72 h, and then the cell's NBT reducing activity was determined by the method of Sakashita et al[24]. with a sligth modification. In brief, the cells were haversted by centrifugation and suspended in 200 ㎕ of 2 ㎎/㎖ NBT solution. After the addition of 2 ㎕ of 100 ㎍/㎖ PMA solution, the cell suspension was incubated at 37℃ for 20 min, 200 ㎕ of 1 N HCl was added at 4℃ to terminate the reaction. After centrifugation, 600 ㎕ of dimethylsulfoxide (DMSO) was added to the cell pellets to solubilize the formazan deposits. The amount of formazan was determined at 570 ㎚ using SpectraMAX 250 microplate spectrophotometer.

2) Flow cytometry

The HL-60 cells exposed to *N. chinensis* were harvested, washed twice with ice-cold PBS, and then suspended in 100 ㎕ of PBS containing 0.25% BSA. After the addition of 10 ㎕ of RPE-conjugated anti-CD11b mAb or FITC-conjugated anti-CD14 mAb, the cells were incubated in the dark at 4℃ for 30 min, washed twice with PBS containing 0.25% BSA, fixed in 500 ㎕ of PBS containing 1% formaldehyde, and then the level of antibody binding to the cells were quantified using fluorescence-activated cell sorting (FACS) Calibur (BD Biosciences, CA, USA).

5. Western blot analysis

Cells were washed with ice-cold phosphate-buffered saline (PBS) and gently resuspend in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium vanadate) with freshly added 1% protease inhibitor cocktail, incubated on ice for 30 min. Cell lysates were centrifuged at 14,000 ×g for 15 min at 4℃, and the protein concentration was determined using a Bradford assay. Samples containing 30 ㎍ of total protein were resolved by a 10% or 12% SDS-PAGE gel, and transferred onto a nitorcellulose membrane for 3 hr at 40V. The membranes were blocked with Tris-buffered saline with Tween-20 (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20) containing 5% milk and probred with different antibodies.

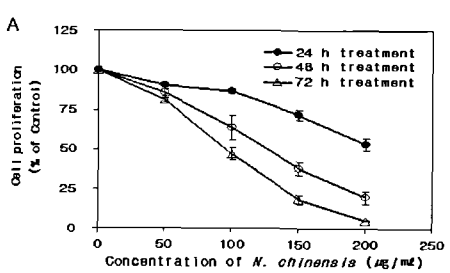
Immunoreactivity was detected using either anti-rabbit or anti-mouse peroxidase-conjugated secondary immunoglobulin G antibody followed by SuperSignal West Pico Chemiluminescent (Pierce, Rockford, IL, USA).

Results

1. Effect of *N. chinensis* on HL-60 cell proliferation and differentiation.

The effect of *N. chinensis* on the cell proliferation and viability was evaluated using the MTT assay. The cells were treated with series of concentrations of *N. chinensis* from 50-200 $\mu g/m\ell$ for 24 h, 48 h, or 72 h. As shown in Fig. 1A, N. chinensis decreased the proliferation of HL-60 cells in dose-dependent manner. After 24 h, 48 h and 72 h, Treatment with 100 $\mu g/m\ell$ of N. chinensis inhibited cell proliferation by 13.8% , 36.5% and 52.9%, respectively, as determined by the MTT assay.

To determine the effect of *N. chinensis* on HL-60 cell differentiation, the HL-60 cells were treated with various concentrations of *N. chinensis* for 72 h and assessed for their NBT reducing activity, which is a marker for degree of cell differentiation. As shown in Fig. 1B, NBT reducing activity was slightly increased about 1.1-fold or 1.5-fold in 10 $\mu g/m\ell$ or 50 $\mu g/m\ell$ of *N. chinensis* treatment, respectively, but markedly increased about 7.5-fold in 100 $\mu g/m\ell$ of *N. chinensis* treatment. To further confirm the cell differentiation induced by *N. chinensis*, the expression of cell surface markers CD11b and CD14 on HL-60 cells were assessed. CD11b (FITC-conjugated) expression was used as a marker of granulocytic and monocytic differentiation, while CD14 (RPE-conjugated) expression was only found in monocytic differentiation. After HL-60 cells were incubated with 100 $\mu g/m\ell$ of *N. chinensis* for 72 h, cell surface markers were immunolabeled and measured by flow cytometry. As shown in Fig 1C, in comparison with the untreated cells, the number of CD11b positive cells in *N. chinensis*-treated HL-60 cells were increased in dose-dependent manner. However, the expression of monocytic CD14 antigen was not increased. These results indicate that *N. chinensis* induced HL-60 cells to undergo granulocytic differentiation.



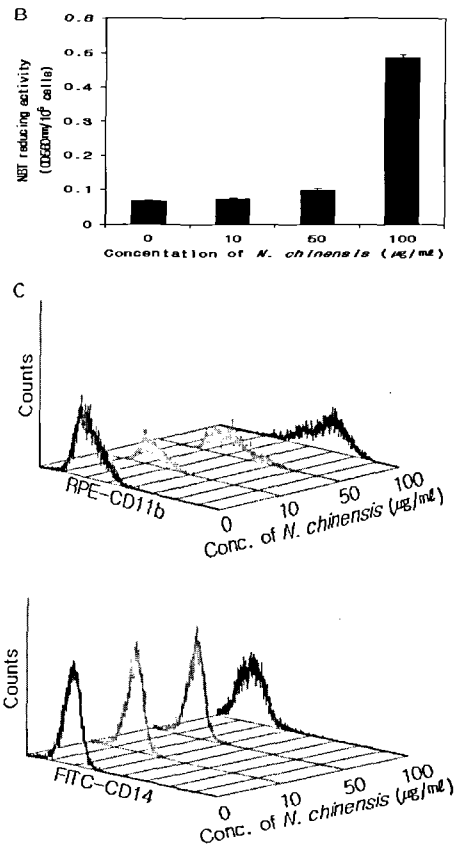


Fig. 1. Effect of *N. chinensis* on proliferation and differentiation of HL-60 cells. The cells were treated with various concentration of *N. chinensis* for 24 h, 48 h, or 72 h. (A) The cell proliferation was determined by the MTT assay. (B) The cellular differentiation was assessed by the NBT reduction assay after 72 h treatment. Value are means ± SD, N = 3. (C) The cells were assessed by FACS analysis using PE-conjugated anti-CD11b mAb or FITC-conjugated anti-CD14 mAb.

2. Effects of *N. chinensis* on activation of MAPKs in HL-60 cells

MAPK signaling pathways have been shown to play an important role in the regulation of differentiation. To clarify the involvement of MAPKs in *N. chinensis*-induced HL-60 cell differentiation, we examined the effects of *N. chinensis* on the activation of ERK, p38 and JNK (Fig. 2). Activation of ERK increased time-dependently in differentiation of HL-60 cells induced by *N. chinensis*. Activation of p38 increased slightly at 24 h after *N. chinensis* treatment, but activation of JNK was unaffected.

To confirm whether activation of ERK and p38 are involved in the on the HL-60 cell differentiation induced by *N. chinensis*, we examined the effect of ERK and p38 inhibitors on *N. chinensis*-induced HL-60 cell differentiation. HL-60 cells were incubated with *N. chinensis* after pretreated with PD98059 (ERK inhibitor) and SB203580 (p38 inhibitor), and then the degree of cellular differentiation was assessed by the NBT reducing activity. As shown in Fig. 3, ERK inhibitor dose-dependently reduced NBT reducing activity in *N. chinensis*-induced HL-60 cell differentiation, indicating that ERK inhibitor inhibited the

cell differentiation induced by *N. chinensis*. In contrast, p38 inhibitor did not inhibit the cell differentiation. These results indicate that ERK pathway may be involved in the HL-60 cell differentiation induced by *N. chinensis*.

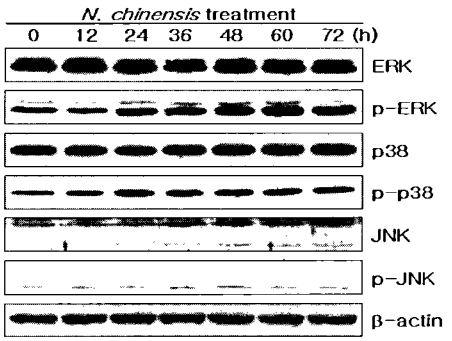


Fig. 2. Effects of *N. chinensis* on activation of ERK, p38 and JNK in HL-60 cells. The cells were treated with *N. chinensis* (100 ㎍/㎖) for 12, 24, 36, 60 and 72 h. Whole cell lysates were subjected to SDS-PAGE followed by Western blot analysis with an anti-ERK, anti-p-ERK, anti-p38, anti-p-p38, anti-JNK and anti-p-JNK antibodies.

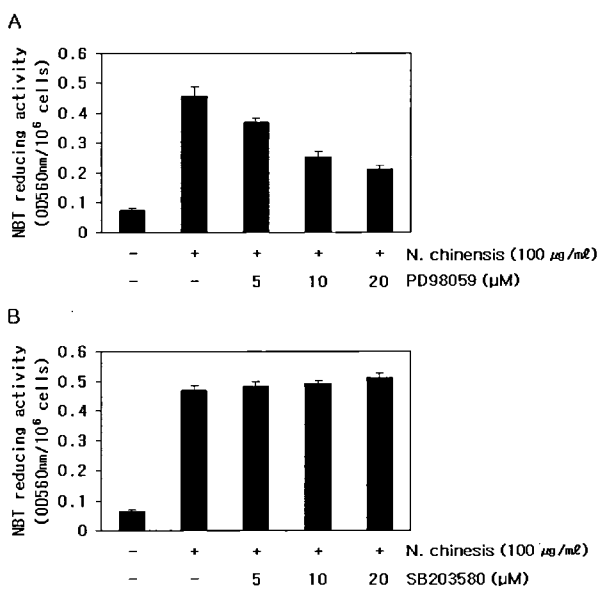


Fig. 3. Effect of inhibitor for ERK and p38 on HL-60 cell differentiation induced by *N. chinensis*. The cells were preincubated with each inhibitor (ERK inhibitor, PD98059; p38 inhibitor, SB203580) for 1 hr, and then treated with 100 ㎍/㎖ *N. chinensis* for 72 hr. The cellular differentiation was assessed by NBT reduction assay. Value are means ± SD, N = 3.

Discussion

In this study, we demonstrated that *N. chinensis* induced differentiation of promyelocytic leukemia HL-60 cells into granulocytes through ERK activation. HL-60 cells provide a convenient system for studying differentiation. HL-60 cells can be induced to differentiate into macrophage/monocyte or granulocyte linages by different inducing chemicals and different cluture conditions. Treatment of HL-60 cells with DMSO or ATRA leads to granulocytic differentiation, while monocytic differentiation can be induced by chemicals such as PMA, 1,25-dihydroxyvitamin D3, or sodium butyrate[25-28].

Induction of HL-60 cell differentiation requires the activation of variety of signal transduction pathways, such as MAPK[19-23] pathways. Recent studies have reported that all-trans retinoic acid (ATRA)-induced granulocytic differentiation activates the ERK pathway[19,20], but not the JNK or p38 pathways. In contrast, ERK pathway had only a transient role in 1,25-dihydroxyvitamin D3-induced monocytic differentiation[21], but activation of the JNK pathway is associated with monocytic differentiation induced by 1,25-dihydroxyvitamin D3[22]. JNK activity also enhanced by inhibition of p38 kinase in monocytic differentiation[23]. Thus, in the ATRA-induced granulocytic differentiation and the 1,25-dihydroxyvitamin D3-induced monocytic differentiation of HL-60 cells, ERK activation is needed for cell differentiation. In our study, activation of ERK and p38 increased in differentiation of HL-60 cells induced by *N. chinensis*, but activation of JNK was unaffected (Fig. 2). However, N. chinensis-induced cell differentiation only reduced by ERK inhibitor (Fig. 3). These results indicates that ERK pathway mediates cellular differentiation in *N. chinensis*-treated HL-60 cells. It is likely that ERK pathway is required for ATRA-induced granulocytic differentiation, but p38 and JNK pathways were not required. ERK activation also lead to growth arrest in monocytic or granulocytic differentiation[19,29]. In previous study, we demonstrated that *N. chinensis* potently inhibits the cell proliferation of HL-60 cells via mechanism of the G1 phase cell cycle arrest through the down-regulation of the CDK2- and CDK6-associated kinase activity in association with the induction of p27Kip[16].

In conclusion, *N. chinensis* markedly increased the activaiton of ERK activation in HL-60 cells. ERK inhibitors also reduced HL-60 cell dfferentiation induced by *N. chinensis*. Taken together, *N. chinensis* induces granulocytic differentiation though ERK activaiton in HL-60 cells. In addition, these results suggest a possible use of *N. chinensis* in the treatment of leukemic diseases.

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