Effect of Nardostachyos Rhizoma on Apoptosis, Differentiation and Proliferation in HL-60 cells

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Nardostachyos Rhizoma (N. Rhizoma) belonging to the family Valerianaceae has been anti-arrhythmic effect, and sedation to the central nerve and a smooth muscle. We reported that the water extract of N. Rhizoma induced apoptotic cell death and differentiation in human promyelocytic leukemia (HL-60) cells. Cytotoxicity of N. Rhizoma was detected only in HL-60 cells (IC50 is about 200 ㎍/㎖). The cytotoxic activity of N. Rhizoma in HL-60 cells was increased in a dose-dependent manner. We used several measures of apoptosis to determine whether these processes were involved in N. Rhizoma-induced apoptotic cell death. The high-dose (200 ㎍/㎖) treatment of N. Rhizoma to HL-60 cells showed cell shrinkage, cell membrane blebbing, apoptotic bodies, and the fragmentation of DNA, suggesting that these cells underwent apoptosis. Treatment of HL-60 cells with N. Rhizoma time-dependently induced activation of caspase-3, caspase-8, and caspase-9 and proteolytic cleavage of poly(ADP-ribose) polymerase. Also, we investigated the effect of N. Rhizoma on cellular differentiation and proliferation in HL-60 cells. Differentiation and proliferation of HL-60 cells was determined through expression of CD11b and CD14 surface antigens using flow cytometry and nitroblue tetrazolium (NBT) assay, and through analysis of cell cycle using propidium iodide assay, respectively. N. Rhizoma induced the differentiation of HL-60 at the low-dose (100 ㎍/㎖) treatment, as shown by increased expression of differentiation surface antigen CD11b, but not CD14 and increased reducing activity of NBT. When HL-60 cells were treated with N. Rhizoma at concentration of 50 ㎍/㎖ and 100 ㎍/㎖, NBT-reducing activities induced approximately 1.5-fold and 20.0-fold as compared with the control. In contrast, HL-60 cells treated with the N. Rhizoma-ATRA combination showed markedly elevated levels of 26.3-fold at 50 ㎍/㎖ N. Rhizoma-0.1 μM ATRA combination and 27.5-fold at 50 ㎍/㎖ N. Rhizoma-0.2 μM ATRA combination than when treated with N. Rhizoma alone or ATRA alone. It may be that N. Rhizoma plays important roles in synergy with ATRA during differentiation of HL-60 cells. DNA flow-cytometry indicated that N. Rhizoma markedly induced a G1 phase arrest of HL-60 cells. N. Rhizoma-treated HL-60 cells increased the cell population in G1 phase from 32.71% to 42.26%, whereas cell population in G2/M and S phases decreased from 23.61% to 10.33% and from 37.78% to 33.98%, respectively. We examined the change in the $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ proteins, which are the CKIs related with the G1 phase arrest. The expression of the CDK inhibitor $p27^{Kip1}$, but not $p21^{WAF1/Cip1}$ were markedly increased by N. Rhizoma. Taken together, these results demonstrated that N. Rhizoma induces apoptotic cell death through activation of caspase-3, and potently inhibits the proliferation of HL-60 cells via the G1 phase cell cycle arrest in association with $p27^{Kip1}$ and granulocytic differentiation induction.

Key words : HL-60, Nardostachyos Rhizoma, Apoptosis, Differentiation, Proliferation, Cell cycle arrest

Introduction

Chemical treatment of the promyelocytic leukemia cell line HL-60 induces differentiation into two cell types of the myeloid lineage: monocyte/macrophage-like cells[1,2] and granulocyte-like cells[3]. 12-O-Tetradecanoyl-phorbol-13-acetate (TPA) and all-trans-retinoic acid (ATRA) are know inducers of HL-60 cells to monocyte/macrophage-[1,2] and granulycote-[4] like phenotypic differentiation, respectively. After exposure to these agents, HL-60 cells stop proliferating and express the phenotypical and physiological functions specific to mature cells.

Cell differentiation is usually associated with exit of the

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cells from the cell cycle. Cell cycle progression in mammalian cells is regulated by a family of enzyme known as cyclin-dependent kinases (CDKs) whose activity is dependent on the binding to specific regulatory subunits called cyclins. The activity of the cyclin/CDK complexes is negatively regulated by specific CDK inhibitors like $p21^{WAF1/Cip1}$ and $p27^{Kip15)}$. The mechanisms regulating cell cycle have a fundamental role in the control of cell proliferation and may also be involved in differentiation.

The differentiation inducers TPA and ATRA have been shown to induce apoptosis in leukemia cells[7,8]. Apoptosis is a form of self-controlled cell death characterized by several morphological changes which differ form those of necrosis and include cell shrinkage, chromatin condensation, membrane blebs and internucleosomal cleavage of DNA[9]. Many tumors result from alterations in the homeostatic control of cell differentiation and apoptosis. However, the relationship between differentiation and apoptosis is still unclear[10]. Apoptosis involves an active participation of the affected cells in cascade of self-destruction cascade that culminates in DNA degradation via endonuclease activation, nuclear disintegration and the formation of 'apoptotic bodies' that involves the cell remnants[11,12]. The induction of apoptosis in proliferating tumor cells may thus be useful as anticancer therapy.

Nardostachyos Rhizoma (N. Rhizoma) belonging to the family Valerianaceae has been in used traditional medicine to elicit stomachic, anti-arrhythmic effect and sedative effect[13]. The plants is known to be rich in sesquiterpenoids[14], which have been found to exhibit antimalarial, antinociceptive[15], and cytotoxic activities[16], as well as to enhance nerve growth factors[17].

In this study, we studied the effects of N. Rhizoma on proliferation and differentiation of the human promyelocytic leukemia cell line, HL-60. We now report that the growth inhibition of human leukemia cells by N. Rhizoma correlates with the induction of differentiation of the leukemia cells into cells of the granulocytic lineage.

Materials and Methods

1. Preparation of Nardostachyos Rhizoma extracts

Nardostachyos Rhizoma was 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 12.8%. 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

Human promyleoid leukemia (HL-60) cell lines used in this study was obtained from Korean Cell Line Bank (KCLB, Seoul, Korea) and routinely cultured under a humidified atmosphere of 5% CO_2-95% air at 37℃ in RPMI 1640 medium (GIBCO BRL) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 200 ㎍/㎖ penicillin and 100㎍/㎖ streptomycin.

3. 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 (Sigma) 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 by ELISA reader (SpectraMAX, Molecular Devices) at 570 ㎚.

4. DNA fragmentation analysis

DNA was purified with the Wizard®Genomic DNA Purification Kit (Promega). Briefly, HL-60 cells (2×10^5 cells/㎖) in 10-Φdishes were treated with various cocentration or time of N. Rhizoma. Treated cells were washed twice with ice-cold PBS and DNA was purified according to some modified manufacturer's protocol and rehydrated in TE buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA) by incubating overnight at 4℃. DNA was analyzed after separation by gel electrophoresis (1.2% agarose gel contained 0.5 ㎍/㎖ethidium bromide at 100 V for 2 h). DNA bands were visualized under UV transilluminator.

5. DAPI staining

The HL-60 cells (2×10^5 cells/㎖) were cultured in 6-well plate in RPMI 1640 medium and HL-60 cells were incubated with various concentrations of N. Rhizoma for 24 h or with 80 ㎍/㎖N. Rhizoma at various time interval. Cells were attached to slides by cytospin, washed with DAPI-methanol (1 ㎍/㎖, Roche), and then stained with DAPI-methanol for 15 min at 37℃. After, stained cells were washed once with methanol and observed under a fluorescence microscope.Apoptotic cells were identified by features characteristic of apoptosis (e.g. nuclear condensation, formation of membrane blebs and apoptotic bodies)

6. Caspase activity assay

Cells were resuspended in lysis buffer (50 mM HEPES,

pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1mM DTT, 0.1 mM EDTA) and lysed by freezing/thawing. Cells lysates were obtained after centrifugation (10,000 rpm) for 1 min at 4℃. Caspase activity assay was performed following the manuufacturer's instructions. In brief, the whole reaction contained 10 ㎕ cell lysates (30 ㎍ total protein), 88 ㎕ reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10mM DTT, 0.1 mM EDTA, 10% glycerol), and 2 ㎕ fluorogenic Ac-DEVD-pNA, Ac-IETD-pNA or Ac-LEHD-pNA (Calbiochem) substrate (200 ㎛M final concentration). Samples were incubated for 3 h at 37℃ and enzyme-catalyzed release of p-nitroanilide was monitored at 405 ㎚ in ELISA reader.

7. Differentiation assay

1) Nitroblue tetrazolium (NBT) reduction assay

HL-60 cells (1 × 10^6/60 ㎜ dish) were cultured with N. Rhizoma, with or without ATRA in RPMI-1640 medium containing 10% FBS for 3 days, and then the cell's NBT reducing activity was determined by the method of Sakashita et al.[18] with a sligth modification. In brief, the cells were haversted by centrifugation and suspended in 200 ㎕ of 2 ㎎/㎖ NBT solution (Sigma). After the addition of 2 ㎕ of 100 ㎍/㎖ TPA solution (Sigma), 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 formed was assayed spectrophotometrically at 560 ㎚ in a spectrophotometer.

2) Flow cytometry the HL-60 cells exposed to N. Rhizoma 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-labled anti-CD 11b or FITC-labled anti-CD 14 antibodies (Dako), 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 FACS flow cytometry.

8. DNA content (FACS) analysis

Cell cycle progression was monitored by quantitating cellular DNA content after staing with propidium iodide (PI). Untreated or N. Rhizoma-treated HL-60 cells were harvested by centrifugation and washed with PBS. The cells were fixed with ice-cold 100% ethanol for 1 h, washed with PBS, and then treated with 10 ㎍/㎖ PI/100 ㎍/㎖ RNase A solution at 37℃ for 1 h in the dark, and analyzed on a fluorescence-activated cell sorter flow cytometer. Cell cycle analysis was determined DNA content from fixed cells stained with PI.

9. Western blot analysis

Cells were washed with ice-cold PBS and gently resuspend in ice-cold lysis buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS) with freshly added 1% protease inhibitor cocktail, incubated on ice for 10 min, and then sonicated three time for 10s on ice. Cell lysates were centrifuged at 14,000 rpm for 10 min at 4℃, and the protein concentration was determined using a Bradford assay. Samples containing 40 ㎍ of total protein were resolved by a 7.5% or 12% SDS-PAGE gel, and transferred onto a nitorcellulose membrane for 3 hr at 40V. Blot were probed with rabbit polyclonal anti-caspase-3, anti-p21 and anti-p27 polyclonal antibodies (Santa Cruz), and mouse monoclonal anti-PARP (BD Pharmingen) antibodies. Immunoreactivity was detected using either and anti-rabbit (Zymed) and anti-mouse (Sigma) peroxidase-conjugated sencondary immunoglobulin G antibody followed by SuperSignal West Pico Chemiluminescent (PIERCE).

Results

1. Cytotoxic effect of N. Rhizoma in HL-60 cells

The effect of N. Rhizoma on the cytotoxicity of cells was evaluated using the MTT assay. Shown as Fig. 1, a 24 h or 48 h exposure to N. Rhizoma dramatically decreased the viability of HL-60 cells in a concentration-dependent manner. The concentration required to inhibit growth of HL-60 cells by 50% (IC_{50}) was approximately 200 ㎍/㎖. The concentration of N. Rhizoma used in this study is 200 ㎍/㎖ (apoptosis experiments) and 100 ㎍/㎖ (differentiation experiments), At the concentration of N. Rhizoma used in differentiation experiments, i.e. 100 ㎍/㎖, cell viability was no significant difference in compared with untreated control.

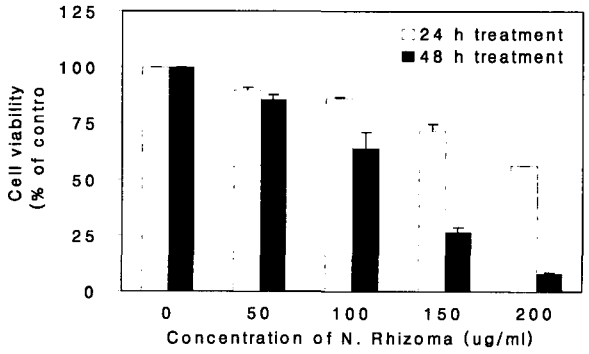

Fig. 1. Effect of N. Rhizoma on cytotoxicity in HL-60 cells. The cells were treated with various concentrations of N. Rhizoma for 24 h or 48 h and the cells were tested for viability by MTT assay. Value are means ± SD, N = 3.

2. N. Rhizoma induces apoptotic cell death in HL-60 cells

The morphology of cells treated with N. Rhizoma was studied to test whether cell death was a result of apoptosis in HL-60 cells. Morphological characteristics of apoptosis, including nuclear condensation and fragmentation, were observed under fluorescent microscope by DAPI staining. The bright blue apoptotic nuclei were readily identified by their condensed chromatin and apoptotic bodies. HL-60 cells were treated for 12 or 24 h with concentration of 200 ㎍/㎖ N. Rhizoma. The apoptotic cells was markedly detected after 12 h of incubation with N. Rhizoma (Fig. 2A). Shown Fig. 2A, along with the appearance of elongated cells, disintegrated cells, as evidenced by apoptotic bodies, and cells with condensed nuclear chromatin appeared in response to N. Rhizoma treatment. The biochemical hallmark of apoptosis in degradation of DNA by endogenous DNase, which cut the internucleosomal regions into double-stranded DNA fragments of 180~200 base pairs. We examined the effects of N. Rhizoma on the internucleosomal DNA fragmentation in HL-60 cells treated for 6, 12, 18 or 24 h with 200 ㎍/㎖ N. Rhizoma by agarose gel electrophoresis. A ladder pattern of internucleosomal DNA fragmentation was observed time-dependent when N. Rhizoma was applied to the HL-60 cells. As shown in Fig. 2B, N. Rhizoma induced DNA fragmentation after treatment for 6 h, and the efficient induction for apoptosis was observed after treatment for 12 h. These results suggest that N. Rhizoma can trigger apoptosis of HL-60 cells.

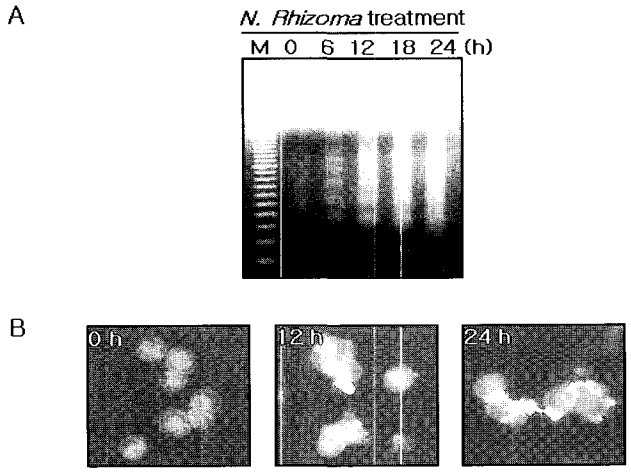

Fig. 2. Effect of N. Rhizoma on DNA fragmentation and morphology in HL-60 cells. (A) The cells were treated with 200 ㎍/㎖ of N. Rhizoma for 0, 6, 12, 18 and 24 h. DNA was extracted, then separated by agarose gel (contained EtBr) electrophoresis, and visualized under UV light. M, 100 bp DNA ladder marker. (B) Cells were treated with 200 ㎍/㎖ of N. Rhizoma for 0, 12 and 24 h. After, cells were subjected to cytospin, stained with DAPI-MeOH and observed under a fluorescence microscope.

3. N. Rhizoma induces activation of caspase-3, -8 and -9

Caspase family plays key roles in the execution of apoptotic cell death. Of these, caspase-3, -8 and -9 are the most important cell executioners for apoptosis. We observed the proteolytic activation of procaspase-3, -8 and -9 induced by the CDST. As shown in Fig 3A, caspase activity assay showed that N. Rhizoma-treatment induced time-dependent activation of caspase-3, -8 and -9. the proteolytic-cleavage of procaspase-3 and -8 reflected the activation of these caspases, Ac-DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA were used as substrates for caspase-3, -8 and -9, respectively. First significant activation of caspases could be detected 3 h after treatment (Fig. 3A). To confirm that the proteolytic-cleavage and activation of procaspase-3, procaspase-3 and PARP have been western blot analysis. N. Rhizoma induced time-dependent cleavage of procaspase-3 (Fig. 3B). Activation of caspase-3 lead to the cleavage of a number of proteins, one of which is poly(ADP-ribose) polymerase (PARP). Although PARP is not essential for cell viability, the cleavage of PARP is another hallmark of apoptosis. As shown in Fig. 3B. treatment of HL-60 cells with various times of N. Rhizoma induced and time-dependent proteolytic cleavage of 116 kDa PARP with accumulation of the 89 kDa cleaved products. Based on these results, we conclude that caspase-3, -8 and -9 plays an important role in N. Rhizoma-induced apoptosis.

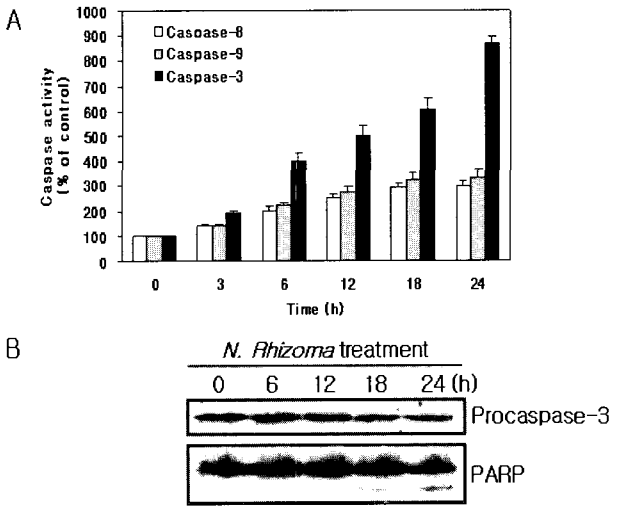

Fig. 3. Effect of N. Rhizoma on activation of caspase-3, -8, -9 and proteolytic cleavage of PARP in HL-60 cells. HL-60 cells were treated with N. Rhizoma (200 ㎍/㎖) for 3, 6, 12, 18, and 24 h. (A) Caspase-3, -8 and -9 activities were measured with AC-DEVD-pNA and AC-IETD-pNA, AC-LEHD-pNA respectively. The activation was measured as the increase in activity (%) with respect to control cells (100%). Value are means ± SD, N = 3. (B) Whole cell lysates were subjected to SDS-PAGE followed by Western blotting with an anti-caspase-3 polyclonal and anti-PARP monoclonal antibodies.

4. Effect of N. Rhizoma on differentiation of HL-60 cells

To determine the effect of N. Rhizoma on cell differentiation, the HL-60 cells were treated with N. Rhizoma, either alone or in combination with ATRA, and the numbers of

differentiated cells, as measured by reducing activity of NBT, were determined. After treatment of HL-60 cells for 72 h, the differentiation inducing activity of N. Rhizoma was assayed compared with ATRA, and the results are shown in Table 1. As shown in Table 1, when HL-60 cells were treated with N. Rhizoma at concentration of 50 ㎍/㎖ and 100 ㎍/㎖, NBT-reducing activities induced approximately 1.5-fold and 20.0-fold as compared with the control, respectively. Also, The differentiation inducing activity of N. Rhizoma were comparable to the activity of ATRA as positive control of differentiation. In contrast, HL-60 cells treated with the N. Rhizoma-ATRA combination showed markedly elevated levels of 26.3-fold at 50 ㎍/㎖ N. Rhizoma-0.1 μM ATRA combination and 27.5-fold at 50 ㎍/㎖ N. Rhizoma-0.2 μM ATRA combination than when treated with N. Rhizoma alone or ATRA alone. It may be that N. Rhizoma plays important roles in synergy with ATRA during differentiation of HL-60 cells. However, N. Rhizoma-ATRA combinations increased cytotoxicity. The induction of HL-60 cell differentiation was also assessed by the expression of cell surface antigens CD11b and CD14. CD11b (FITC-labelled) expression was used as a marker of granulocytic and monocytic differentiation, while CD14 (RPE-labelled) expression was only found in monocytic differentiation. After HL-60 cells were incubated with or without 12 μM N. Rhizoma for 72 h, cell surface markers were immunolabeled and measured by flow cytometry. As shown in Fig 4, in comparison with the untreated cells, the amount of CD11b positive cells in N. Rhizoma-treated HL-60 cells were increased significantly (60.87%) in compared with untreated control (3.21%). However, the expression of monocytic CD14 antigen was not significantly increased. These results indicate that N. Rhizoma induced HL-60 cells to undergo granulocytic differentiation.

Table 1. Effects of N. Rhizoma with or without ATRA on proliferation and differentiation of HL-60 cells

Compound	Cell proliferation[a] (% of control)	NBT reduction[b] (A560/10^5 cells)
Control	100	0.04±0.003
0.1 μM ATRA	62.3±1.4[c]	0.23±0.009[c]
0.2 μM ATRA	61.6±0.7[c]	0.31±0.019[c]
50 ㎍/ml NRWE	69.2±1.8[c]	0.06±0.002
100 ㎍/ml NRWE	53.9±2.1[c]	0.80±0.049[c]
0.1 μM ATRA +50 ㎍/ml NRWE	37.5±3.3[c]	1.05±0.126
0.2 μM ATRA +100 ㎍/ml NRWE	28.6±9.9	1.10±0.078[c]

a Cell proliferation was determined by MTT assay after 96 h treatment. b NBT reducting activity was determined after 96 h treatment. c P<0.05, significantly different from the control. Value are means ± SD, N = 3

5. Effect of N. Rhizoma on the cell cycle distribution in HL-60 cells

The effect N. Rhizoma on the cell cycle progression in HL-60 cells was determined by FACS. The ATRA, a differentiating agent, inhibit cell proliferation and arrest the cells in G0/G1 phase. In a previous study, it was suggested that the maintenance of G0/G1 phase might be necessary for HL-60 granulocytic differentiation. HL-60 cells were incubated with or without 100 ㎍/㎖ N. Rhizoma for 72 h and analyzed by DNA flow cytometry. As shown in Fig. 5, N. Rhizoma-treated HL-60 cells increased the cell population in G1 phase from 32.71% to 42.26%, whereas cell population in G2/M and S phases decreased from 37.78% to 33.98% and from 23.61% to 10.33%, respectively. As a result, DNA flow cytometric analysis indicate that HL-60 cells induced a G1 phase arrest of cell cycle after N. Rhizoma treatment.

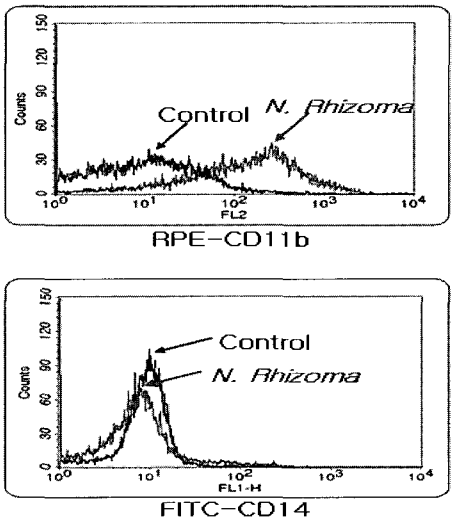

Fig. 4. The differentiation-inducing effect of N. Rhizoma. FACS analysis of the expression of the CD11b and CD14 antigens in HL-60 cells treated with 100 ㎍/ml N. Rhizoma for 72 h.

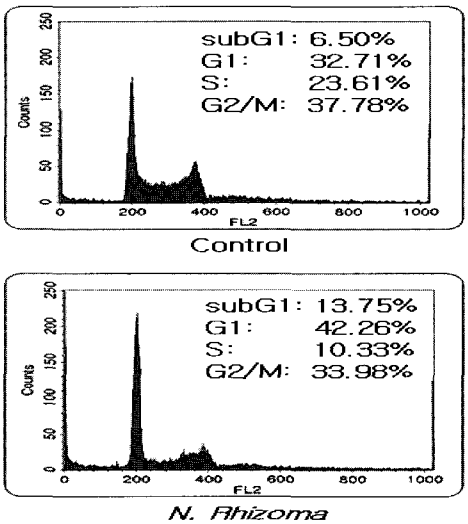

Fig. 5. The effect of N. Rhizoma on the DNA content from the HL-60 cells. The cells were exposed to 100 ㎍/ml N. Rhizoma for 72 h, wahsed and then harvested. The cells were fixed and stained with PI and the DNA content was analyzed by FACS.

6. Effect of N. Rhizoma on p21WAF1/Cip1 and p27Kip1 expression in HL-60 cells

Because N. Rhizoma induced a G1 arrest in HL-60 cells, we next examined the change in the $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ proteins, which are the CKIs related with the G1 phase arrest. $p21^{WAF1/Cip1}$ protein acts as a downstream mediator of the tumor suppressor p53 that functions as the G1 phase checkpoint, resulting in G1 arrest. $p27^{Kip1}$, a member of a family of proteins that includes $p21^{WAF1/Cip1}$ and $p57^{Kip2}$, is a universal CDK inhibitor that negatively regulates G1 CDKs. After N. Rhizoma treatment in HL-60 cells, the $p27^{Kip1}$ protein level increased in a time-dependent manner, whereas no detectable change was observed in the level the $p21^{WAF1/Cip1}$ (Fig. 6). As shown in Fig. 6, the level of $p27^{Kip1}$ began to increase slightly after 24 h incubation with N. Rhizoma and had clearly increased at 48 h. These results suggested that induction of $p27^{Kip1}$ protein, but not $p21^{WAF1/Cip1}$ were involved in G0/G1 phase arrest of N. Rhizoma-treated HL-60 cells.

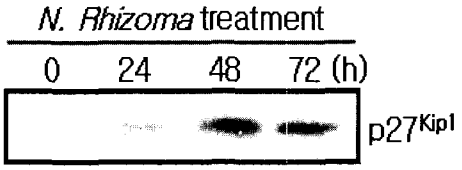

Fig. 6. The effect of N. Rhizoma on the expression of p27Kip1 proteins from the HL-60 cells. The cells were harvested at the indicated times after incubation with 100 ㎍/㎖ N. Rhizoma. Cells were the lysed, and the supernatants were subjected to Western blot analysis using anti-p27Kip1 antibody.

Discussion

This study demonstrates that N. Rhizoma potently induces the apoptotic cell death and causes the cell cycle arrest in the HL-60 cells, leading to the inhibition of the cell proliferation and the induction of differentiation. We have shown that N. Rhizoma was cytotoxic to HL-60 cells; the IC_{50} value was approximately 200 ㎍/㎖ (Fig. 1). Analysis of cell morphology, DNA fragmentation, and caspase activity in HL-60 cells incubated with N. Rhizoma suggest that the cytotoxicity of N. Rhizoma was mediated by the induction of apoptosis. Apoptosis is an important phenomenon in cancer chemotherapy, because anticancer drugs exert their antitumor effect against cancer cells by inducing apoptosis. The general feature of apoptosis is apoptotic body and DNA fragmentation. Typically, the apoptotic body was characterized by nuclear condensation and cell shrinkage, and DNA fragmentation was formed by cleavage of DNA of apoptotic cells, which is population of multimers of 180~200 bp fragments. After HL-60 cells were treated with N. Rhizoma for various times, we have observed apoptotic bodies in morphological analysis of apoptotic cells with DAPI staining (Fig. 2A) and confirmed DNA fragment ladder formation through gel electrophoresis (Fig. 2B).

Apoptosis is mainly brought about by activation of caspases, a protease family with unique substrate selectivity. It is known that caspase-3, which is the main executioner caspase, can be activated by caspase-8 and/or caspase-9[19]. It depends upon the apoptosis-inducing stimulus whether caspase-3 is activiated by both/either caspase. As shown in Fig. 3, N. Rhizoma have increased the caspase-3 by both caspase-8 and caspase-9, but how N. Rhizoma activates caspase-3 is not clear. Because activation of caspase-3 mediated by two pathways consisting of the initiator caspases, caspase-8 or caspase-9. Caspase-8 can activate apoptotic pathways involving effector caspases through mitochondria-dependent or-independent pathways. In the mitochondria-independent pathway, caspase-8 can directly activate caspase-3, but in the mithochondria -dependent, which involves the release of cytochrome c from mitochondria, activate caspase-3 through induction of caspase-9[20,21]. Apoptotic pathway of HL-60 cells by N. Rhizoma is not yet clear. We are need of further studies that examine the signaling pathway of apoptotic cell death by N. Rhizoma.

The present study has demonstrated tha N. Rhizoma exerts a potent differentiation-inducing activity on HL-60 cells. This effect of N. Rhizoma was confirmed with a NBT reduction assay and expression of cell surface antigens. In NBT reduction assay, combination of N. Rhizoma and ATRA synergically increased than when treated with N. Rhizoma alone or ATRA alone, whereas cell viability has reduced (Table. 1). Because this will probably induced terminal differentiation after short-period of time by synergical effect of N. Rhizoma-ATRA combination. Thus, reduction of cell viability may be caused that terminal differentiation is usually followed by cell death via apoptosis[7,22]. Differentiation induced by N. Rhizoma increased expression of cell surface antigen CD11b, but not CD14 (Fig. 4). Phenotypic cell surface antigen CD11b is a surface marker for granulocytes. Thus, N. Rhizoma was concluded to have the ability to induce the differentiation of HL-60 cells into mature cells, those of granulocytic lineage. Cell differentiation is regulated in a cell cycle-dependent manner. For example, the differentiation of hematopoietic cells is associated with a loss of cell cycling capacity, and the cells become arrested in the G0/G1 phase of the cell cycle[23]. The cell cycle analysis revealed that N. Rhizoma could markedly induce a G1 phase arrest in HL-60 cells, but had a reduction effect on the G2/M and S phases (Fig. 5). Although the mechanism of differentiation induction by N. Rhizoma is not clear, it may be that $p27^{Kip1}$ plays an important role in

modulation of the cell differentiation activity. Inhibition of the G1/S transition induces growth arrest and granulocyte differentiation of HL-60 cells, which is mediated by a block of cell cycle progression at the G1 phase[24,25]. The $p27^{Kip1}$ protein is a cycline-dependent kinase (CDK) inhibitor that is one of the regulators of cell cycle progression in the G1/S transition[26]. Exposure of HL-60 cells to the N. Rhizoma increased expression of $p27^{Kip1}$ protein (Fig. 6). Taken together, $p27^{Kip1}$ induction occurs through a G1 arrest mechanism, and they raise the possibility that differentiation-associated stimuli may be closely associated with the terminal differentiation of promyeloid leukemic cells.

In conclusion, N. Rhizoma induces apoptotic cell death through activation of caspase-3, and potently inhibits the proliferation of HL-60 cells via the G1 phase cell cycle arrest in association with $p27^{Kip1}$ and granulocytic differentiation induction. Finally, these results suggest that N. Rhizoma may be useful to the investigation for leukemia therapy.

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