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Effects of *Aralia continentalis* Root Extract on Cell Proliferation and Apoptosis in Human Promyelocytic Leukemia HL-60 Cells

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Abstract The roots of *Aralia continentalis* (AC) have been used traditionally in Korean as a folk medicine for anti-inflammation and as an anti-rheumatic. In this study, we report that the ethyl acetate-soluble fraction (ACE) of the methanolic extract of AC root inhibited the cell growth of various human cancer cell lines and induced apoptosis of HL-60, human promyelocytic leukemia cells. Its IC_{50} values on growth inhibition were estimated to be 56.3 μg/ml on HL-60, 87.2 μg/ml on HepG2, 93.2 μg/ml on HeLa, 135.5 μg/ml on DU-145, and 135.8 μg/ml on HT-29 cells. Interestingly, ACE showed no antiproliferative effect on normal lymphocyte cells used as control. Furthermore, nuclear DAPI staining revealed the typical nuclear features of apoptosis in the HL-60 cells exposed to 80 μg/ml ACE, and a flow cytometric analysis of the HL-60 cells using propidium iodide showed that the apoptotic cell population increased gradually from 5% at 0 h to 16% at 12 h and 20% at 24 h after treated with 50 μg/ml of ACE. TUNEL assay also revealed the apoptotic induction of the HL-60 cells treated with ACE. To obtain further information on the ACE-induced apoptosis, the expression level of certain apoptosis-associated proteins was examined using a Western blot analysis. Treatment of the HL-60 cells with ACE resulted in the activation of caspase-3, and subsequent proteolytic cleavage of poly(ADP-ribose) polymerase (PARP). The above results confirmed that the apoptosis in the HL-60 cells was induced by ACE, and that caspase-3-mediated PARP cleavage was involved in the process.

Key words: *Aralia continentalis*, Dokwhal, cell growth inhibition, apoptosis

Apoptotic cell death has been characterized as a fundamental cellular activity to maintain the physiological balance of the organism. It is also involved in immune defense machinery and plays a necessary role as a protective mechanism against carcinogenesis by eliminating damaged cells or excess cells abnormally proliferated [1, 7]. Emerging evidence has demonstrated that the anticancer activities of certain chemotherapeutic agents are involved in the induction of apoptosis, which is considered to be a preferred way to manage cancer [1, 7].

Medicinal plants have been used for the treatment of various tumors for centuries. Recently, bioassay-guided investigations on plants used in traditional medicine have led to the discovery of many new antitumor agents [12–15]. The root of *Aralia continentalis* (AC) Kitagawa (Korean name, "Dokwhal") has long been used as a folk medicine in Korea for the treatment of various diseases including as an anti-rheumatic, anti-inflammation, and analgesic [16]. The anti-inflammatory activities of several diterpenoic acids isolated from AC have been reported [5, 6]; however, scientific approach for these antiproliferative and apoptotic effects has not yet been carried out. Accordingly, the current study was undertaken to examine the antiproliferative effects of the ethyl acetate-soluble fraction (ACE) of the AC root methanolic extract on several human cancer cell lines and to characterize ACE-induced cell apoptosis of human promyelocytic leukemia HL-60 cells, by studying apoptotic morphological changes, chromatin condensation, DNA fragmentation, activation of caspase-3, and proteolytic cleavage of PARP. It should be noted that the HL-60 cell line has been widely used as a model system for testing anti-leukemic as well as general anti-tumor agents [24] and also in studies on induction and mechanisms of cancer cell apoptosis [8, 24].

MATERIALS AND METHODS

Chemicals

Phosphate-buffered saline (PBS), MEM, and RPMI 1640 media were purchased from GIBCO, Ltd. (Grand Island,

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NY, U.S.A.), whereas the mouse monoclonal antibodies against poly(ADP-ribose) polymerase (PARP), caspase-3, and β-actin were all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden) and the protein assay kit from Bio-Rad Laboratories (Hercules, CA, U.S.A.). All other materials were obtained from Sigma (St. Louis, MO, U.S.A.).

Cell Line and Cell Culture

Human cervical adenocarcinoma cell line (HeLa), human hepatocellular carcinoma cell line (HepG2), human promyelocytic leukemia cell line (HL-60), human prostate carcinoma cell line (DU-145), and human colon cancer cell line (HT-29) were all obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The cells were cultured in MEM (for HeLa) and RPMI 1640 (for HepG2, HL-60, HT-29, and DU-145) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The cell density in the culture did not exceed 1×10^6 cells/ml.

Preparation of ACE

The roots of AC were collected from Kyungdong market in Seoul and stored at −70°C before use. Fresh and dried roots were pounded into granules with an electrical mill and then extracted with 1 l of 70% methanol at 80–85°C for 6 h. The methanolic extract was filtered through a filter paper (Advantec No.1, Japan) and then concentrated *in vacuo* until the total volume reached 100 ml. The concentrated extract was partitioned three times into water and ethyl acetate, and each phase was then evaporated to dryness under vaccum. The maximum growth inhibition of various cancer cell lines was demonstrated by the ethyl acetate-soluble portion (ACE), using an MTT assay.

MTT Assay

The antiproliferative effect of ACE was determined by MTT assay [18], using an MTT reagent kit purchased from Sigma Chemical (St. Louis, U.S.A.), and viable cells were counted. Thus, various human cancer cell lines (1.4×10^5 cells per well) were seeded in a 96-well plate in 100 μl of the cell culture medium. After incubation for 24 h, 1 μl that contained various concentrations (0–200 μg/ml) of ACE was added to the cancer cells to be tested, with normal lymphocytes as a control. After incubation at 37°C for 24 h, the culture medium was carefully removed without disturbing the cells and replaced with 100 μl of a fresh cell medium. Subsequently, 15 μl of MTT reagent was added to each well, and the plates were incubated again in a CO_2 incubator at 37°C for 3 h. Thereafter, the supernatant was removed from each well, and 100 μl of DMSO was then added to dissolve the colored formazan crystals produced by MTT. Subsequently, optical density at 570 nm was measured using the ELISA Reader (Molecular Devices Corp., Sunnyvale, U.S.A.).

Nuclear Staining with DAPI

HL-60 cells (1×10^6 cells /ml) were cultured in an RPMI 1640 medium containing 10% FBS in the absence or presence of ACE (80 μg/ml). After 48 h of incubation, the cells were harvested, washed with PBS, and then 4% neutral-buffered formalin (100 μl) was added to the cell pellet. Next, an aliquot (50 μl) of the cell suspension was smeared on slides and dried at room temperature (RT), and then the fixed cells were washed in PBS, air dried, and stained with DNA-specific fluorochrome DAPI for 30 min at 37°C. Finally, the slides were observed under a fluorescence microscope.

Propidium Iodide Staining for Flow Cytometric Analysis

HL-60 cells (1×10^6 cells /ml) were treated without or with 50 μg/ml ACE and harvested at indicated times (0, 3, 6, 9, 12, 24 h). After washing twice with ice-cold PBS, the cells were fixed with ice-cold PBS in 70% ethanol and stored at 4°C. For a flow cytometric analysis, the cells were incubated with 0.1 mg/ml RNase A at 37°C for 30 min, stained with 50 μg/ml propidium iodide (PI) for 30 min on ice, and then measured using a FASTAR flow cytometer (Becton Dickinson, San Diego, U.S.A.) with Cell Quest software.

TUNEL Assay for Detection of DNA Fragmentation

The detection of apoptotic cells was carried out according to the manufacturer's protocol, using an Apoptosis Detection System, Fluorescein (Promega Corp., Madison, U.S.A.). HL-60 cells (1×10^6 cells/ml) were incubated in RPMI 1640 medium with 10% FBS for different times after treatment with 80 μg/ml of ACE. The HL-60 cells were then washed in PBS and fixed in 1% formaldehyde solution for 20 min on ice. Thereafter, the fixed cells were washed with cold 70% ethanol, and the dehydrated cells were incubated for 4 h at −20°C. The cells were then washed again in PBS and resuspended in an equilibration buffer for 5 min at RT, followed by TdT reaction buffer (50 μl) at 37°C for 60 min. After terminating the TdT reaction, the cells were incubated in 1 ml of PBS, containing 25 μg/ml PI and 250 μg/ml RNase A, at RT for 30 min in the dark. The fluorescein-12-dUTP-labeled DNA was quantitated using a FASTAR flow cytometer with Cell Quest software.

Western Blot Analysis

HL-60 cells were plated onto 60-mm dishes at a density of 2×10^5 cells/ml without or with ACE (80 μg/ml) and then harvested at indicated times. To prepare the whole-cell extract, the cells were washed with PBS

and suspended in protein lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 100 μg/ml PMSF, and protease inhibitors). The protein content was determined with Bio-Rad protein assay reagent, using bovine serum albumin as the standard. The protein extracts (30–50 μg) were then resolved on 8–14% SDS-PAGE and transferred to a PVDF membrane (Millipore, U.S.A.). The membranes were blocked with 5% w/v nonfat dry milk, and then incubated with the indicated antibodies in Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl, pH 7.6) containing 0.1% Tween-20 with gentle shaking at 4°C for 2–12 h. Peroxidase-conjugated goat anti-mouse antibody was used as the secondary antibody. The signals were detected using an ECL Western blotting kit (Amersham Pharmacia Biotech).

RESULTS

Growth Inhibitory Effect of ACE on Human Cancer Cells and Normal Lymphocytes

The cell growth inhibitory effect of ACE on five human cancer cell lines and normal lymphocytes as a control was determined by assessing the viability of the cells, using an MTT assay. Thus, HeLa, HepG2, HL-60, DU-145, HT-29, and normal lymphocyte cells were all cultured in varying concentrations of ACE for 24 h, and all assays were performed in triplicate. As shown in Fig. 1, the growth of all of the cancer cells tested was inhibited by ACE in a concentration-dependent manner. The estimated IC_{50} for ACE in these cancer cell lines ranged from 56 to 136 μg/ml. Interestingly, however, ACE showed no antiproliferative effect on the normal lymphocyte cells used as control.

Induction of Apoptosis in HL-60 Cells by ACE

To evaluate whether the growth inhibition of the HL-60 cells by ACE was related to apoptosis, the cells were first subjected to 80 μg/ml ACE and their morphological changes were examined by nuclear DAPI staining for 48 h. As illustrated in Fig. 2, the cells exposed to ACE exhibited apoptotic features, such as DNA condensation and DNA fragmentation (Fig. 2B). This apoptotic morphological change was also confirmed with a flow cytometry analysis, where PI staining of the HL-60 cells treated with ACE

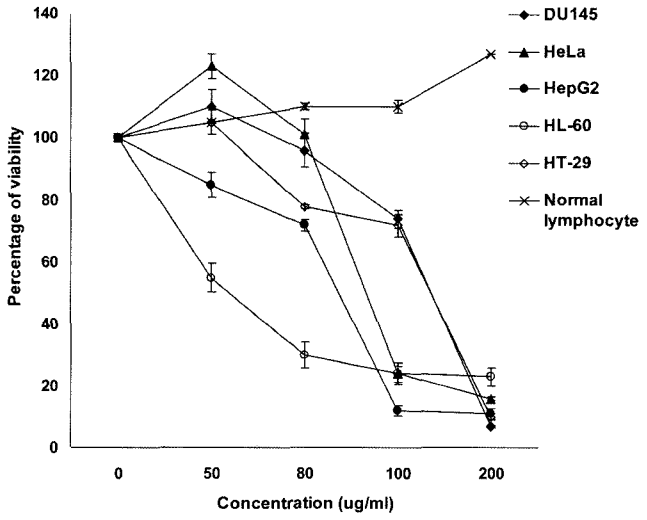

Fig. 1. Effect of ACE on growth of various human cancer cells and normal lymphocytes determined by MTT assay.
The cells were treated with various concentrations of ACE for 24 h, as described in the text. The results represent mean±SD of three independent experiments. IC_{50}: 56.3 μg/ml in HL-60 cells, 87.2 μg/ml in HepG2 cells, 93.2 μg/ml in HeLa cells, 135.5 μg/ml in DU-145 cells, and 135.8 μg/ml in HT-29 cells.

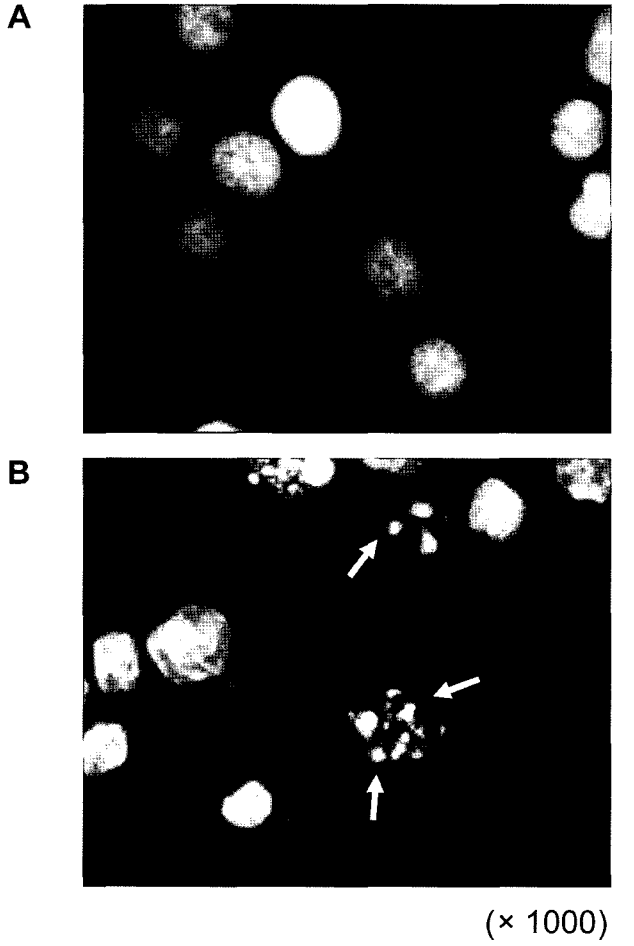

Fig. 2. Morphologic changes of the HL-60 cells induced by ACE.
The cells were cultured in the absence (**A**) or presence (**B**) of 80 μg/ml ACE for 48 h before the cells were fixed and stained with DAPI. The stained nuclei were then observed under a fluorescent microscope (magnification, 1,000×). The arrows indicate apoptotic bodies of nuclear fragmentation.

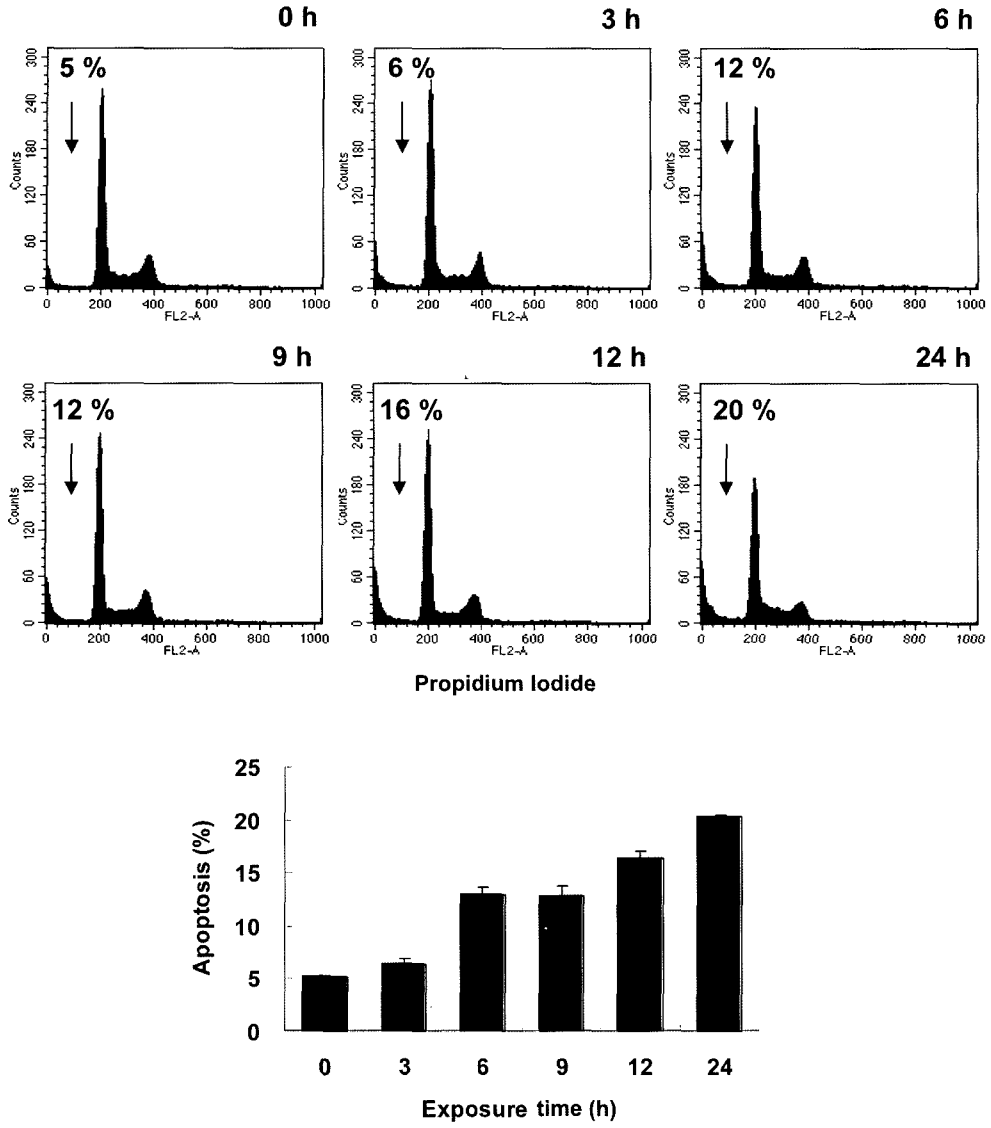

Fig. 3. Quantification of apoptosis by flow cytometry.
The HL-60 cells were treated with 50 μg/ml ACE for the indicated times. The cells were then stained with propidium iodide, and nuclei were analyzed for their DNA content using flow cytometry with Cell Quest software. A total of 10,000 nuclei were analyzed from each sample. Data are mean±SD of three separate experiments.

revealed that the apoptotic cell population increased gradually: 5%, 12%, 16%, and 20% at 0 h, 6 h, 12 h, and 24 h, respectively, after exposure to 50 μg/ml ACE (Fig. 3). Moreover, as seen in Fig. 4, the TUNEL assay showed that, when the HL-60 cells were incubated with 80 μg/ml of ACE for 24 or 48 h, apoptotic DNA fragmentation was observed in the cells.

Effect of ACE on Caspase-3 Activation and PARP Cleavage

To determine the expression level of apoptosis-related proteins induced in the ACE-treated HL-60 cells, caspase-3 and PARP were investigated by Western blot analysis. As shown in Fig. 5, Western blot revealed a time-dependent decrease of procaspase-3 and its cleavage into an active form. It is known that the activation of caspase-3 leads to the cleavage of a number of proteins, one of which is PARP. Although PARP is not essential for cell death, the cleavage of PARP is an another hallmark of apoptosis. Furthermore, PARP is characteristically processed during apoptosis from its native 116 kDa form into a truncated 85 kDa product [3]. In the present study, the treatment of the HL-60 cells with ACE caused a time-dependent proteolytic cleavage of PARP. Therefore, taken together, the results clearly showed that the ACE-induced apoptosis involved the activation of caspase-3.

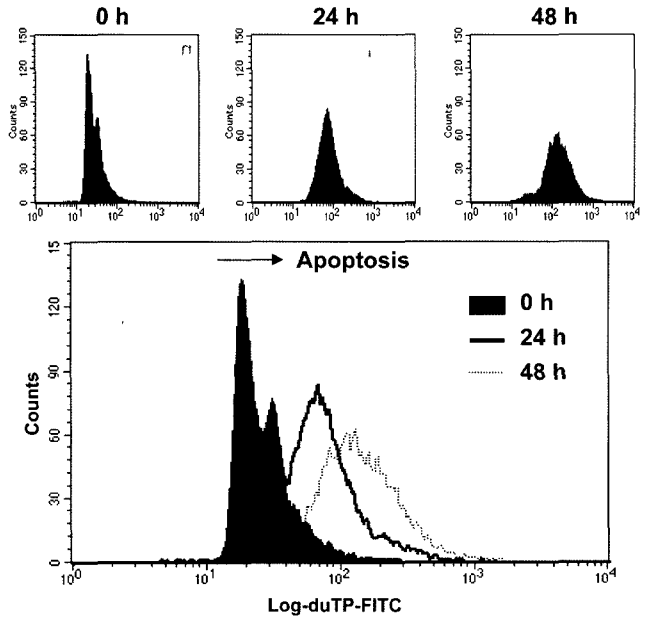

Fig. 4. Induction of apoptosis by ACE in the HL-60 cells, as determined by TUNEL assay.
The cells were incubated for 24 and 48 h in 80 μg/ml of ACE, and then fixed, permeabilized, and stained with fluorescent TUNEL reaction (Promega). Thereafter, the cells were analyzed using flow cytometry.

DISCUSSION

Induction of apoptosis, programmed cell death, is one approach to cancer therapy [11, 17]. Apoptotic cell death is a physiological mechanism that eliminates unwanted cells by triggering the cell's intrinsic suicide program [10]. Impairment of the apoptotic mechanism ultimately generates pathological conditions including developmental defects such as autoimmune diseases, neurodegeneration, or cancerous neoplasia [20]. Apoptosis is characterized by cellular morphological changes, chromatin condensation, and apoptotic bodies associated with DNA cleavage into ladders [9, 24]. These changes result from the proteolytic cleavage of various intracellular polypeptides, mostly due to a family of cysteine proteases called caspases. Caspases are normally present in the cell as proenzymes and require limited proteolysis for the activation of enzyme activity [19]. The sequential activation of caspases from upstream to downstream of the apoptosis signal pathway is thus required for the initiation and execution of apoptosis. Caspase-3 is a major downstream effector of apoptosis, and the caspase-3-mediated proteolytic cleavage of PARP and other substrates is a critical step that leads to subsequent DNA fragmentation [4], nuclear condensation [21], and membrane blebbing [2, 22]. All these major apoptotic events were detected in the ACE-treated HL-60 cells. Namely, the present study revealed that ACE had a growth inhibitory effect on various human cancer cells, which appeared to be associated with the induction of apoptosis (Fig. 1). Interestingly, however, ACE showed no antiproliferative effect on the normal lymphocyte cells used as the control. The ACE-induced apoptosis was confirmed by nuclear morphologic changes and DNA fragmentation (Fig. 2), and flow cytometric analysis (Figs. 3 and 4). Furthermore, Western blot analysis revealed a time-dependent decrease of procaspase-3 and PARP cleavage in the ACE-treated HL-60 cells (Fig. 5). Therefore, the results confirmed the ACE-induced apoptosis in HL-60 cells and the involvement of caspase-3-mediated PARP cleavage in the process.

In conclusion, the present results strongly suggest that ACE isolated from *Aralia continentalis* may contain a bioactive compound that kills human promyelocytic leukemia cells, HL-60 cells, by an apoptosis mechanism via the activation of caspase-3, thus suggesting a potential therapeutic role of these herbal ingredients against HL-60 cells.

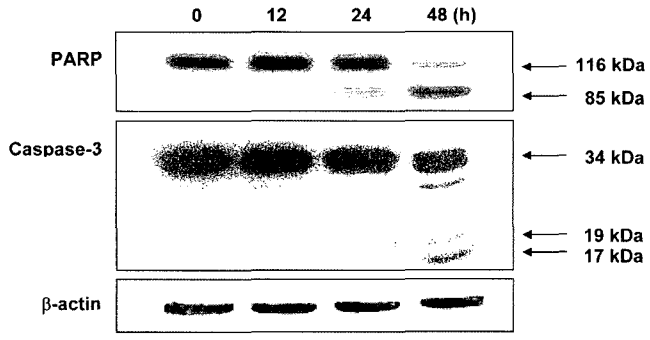

Fig. 5. Effect of ACE on activation of caspase-3 and cleavage of PARP.
Total cell lysates from the HL-60 cells treated with 80 μg/ml ACE for the indicated times were electrophoretically separated on 8–14% polyacrylamide gel and immunoblotted with an antibody against each protein and β-actin that served as the internal control.

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REFERENCES

1. Brown, J. M. and B. G. Wouters. 1999. Apoptosis, p53, and tumor cell sensitivity to anticancer agents. *Cancer Res.* **59:** 1391–1399.
2. Coleman, M. L., E. A. Sahai, M. Yeo, M. Bosch, A. Dewar, and M. F. Olson. 2001. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. *Nat. Cell Biol.* **3:** 339–345.

3. Dantzer, F., V. Schreiber, C. Niedergang, C. Trucco, E. Flatter, G. De La Rubia, J. Oliver, V. Rolli, J. Ménissier-de Murcia, and G. de Murcia. 1999. Involvement of poly(ADP-ribose) polymerase in base excision repair. *Biochimie* **81:** 69–75.
4. Enari, M., H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, and S. Nagata. 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391:** 43–50.
5. Han, B. H., Y. N. Han, K. A. Han, M. H. Park, and E. O. Lee. 1983. Studies on the anti-inflammatory activity of *Aralia continentalis* (I). *Arch. Pharm. Res.* **6:** 17–23.
6. Han, B. H., E. R. Woo, M. H. Park, and Y. N. Han. 1985. Studies on the anti-inflammatory activity of *Aralia continentalis* (III). *Arch. Pharm. Res.* **8:** 59–65.
7. Hengartner, M. O. 2000. The biochemistry of apoptosis. *Nature* **407:** 770–776.
8. Kaufman, S. H. 1989. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin and other cytotoxic anticancer drugs: A cautionary note. *Cancer Res.* **49:** 5870–5878.
9. Kaufman, S. H. and W. C. Earnshaw. 2000. Induction of apoptosis by cancer chemotherapy. *Exp. Cell Res.* **256:** 42–49.
10. Kerr, J. F. R., A. H. Wyllie, and A. R. Currie. 1972. Apoptosis: A basic biological phenomenon with wide-ranging implication in tissue kinetics. *Brit. J. Cancer* **26:** 239–257.
11. Kim, M., C. Shin, H. Yang, S. Kim, H. Lim, C.-H. Lee, M. Kim, and Y. Lim. 2004. Naltriben analogues as peptide anticancer drugs. *J. Microbiol. Biotechnol.* **14:** 881–884.
12. Lee, C.-H., H. Lim, M. Kim, Y. Cho, D. Oh, C. Kim, and Y. Lim. 2002. Isolation and biological properties of novel cell cycle inhibitor, HY558, isolated from *Penicillium minioluteum* F558. *J. Microbiol. Biotechnol.* **12:** 470–475.
13. Lee, H.-K., I.-H. Lee, J.-S. Yim, Y.-H. Kim, S.-H. Lee, K.-S. Lee, Y.-M. Koo, S.-J. Kim, and B.-C. Jeong. 2005. Purification and characterization of CDMHK, a growth inhibitory molecule against cancer cell lines, from *Myxobacterium* sp. HK1 isolated from Korean soil. *J. Microbiol. Biotechnol.* **15:** 734–739.
14. Lim, H., M. Kim, Y. Cho, J. Kim, Y. Lim, and C.-H. Lee. 2004. Inhibition of cell cycle progression and induction of apoptosis in HeLa cells by HY558-1, a novel CDK inhibitor isolated from *Penicillium minioluteum* F558. *J. Microbiol. Biotechnol.* **14:** 978–984.
15. Lim, H., M. Kim, Y. Lim, Y. Cho, and C.-H. Lee. 2006. Inhibition of cell-cycle progression in HeLa cells by HY52, a novel cyclin-dependent kinase inhibitor isolated from *Bauhinia forficata*. *Cancer Lett.* **233:** 89–97.
16. Lim, T. Y. 1987. Comparative study on the pharmacological effect of kinds of Dokhwal. *J. Kor. Orient. Med.* **8:** 97–98.
17. Los, M., C. J. Burek, C. Strol, K. Bnedyk, H. Hug, and A. Mackiewicz. 2003. Anticancer drugs of tomorrow: Apoptotic pathways as targets for drug design. *Drug Discov. Today* **8:** 67–77.
18. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65:** 55–63.
19. Nunez, G., M. A. Benedict, Y. Hu, and N. Inohara. 1998. Caspases: The proteases of the apoptotic pathway. *Oncogene* **17:** 3237–3245.
20. Reed, J. C. 2001. Apoptosis regulatory proteins as targets for drug discovery. *Trends Mol. Med.* **7:** 314–318.
21. Sahara, S., M. Aoto, Y. Eguchi, N. Imamoto, Y. Yoneda, and Y. Tsujimoto. 1999. Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation. *Nature* **401:** 168–173.
22. Sebbagh, M., C. Renvoize, J. Hamelin, N. Riche, J. Bertoglio, and J. Breard. 2001. Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. *Nat. Cell Biol.* **3:** 346–352.
23. Suh, N., L. Luyengi, H. H. Fong, A. D. Kinghorn, and J. M. Pezzuto. 1995. Discovery of natural product chemopreventive agents utilizing HL-60 cell differentiation as a model. *Anticancer Res.* **15:** 233–239.
24. Studzinski, G. P. 1999. An overview of apoptosis. *In* G. P. Studzinski (ed.), *Apoptosis: A Practical Approach*. Oxford University Press, Oxford, U.K.