Arch Pharm Res Vol 34, No 4, 625-633, 2011
DOI 10.1007/s12272-011-0413-8

Archives of Pharmacal Research
www.springer.com/12272

Anticancer Potential of Magnolol for Lung Cancer Treatment

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(Received October 12, 2010/Revised November 29, 2010/Accepted December 29, 2010)

Lung malignancy is a major cause of human mortality. As such, safe pharmacological agents that can detect lung cancer are urgently required. Magnolol has been reported to have anticancer property. However, it is still unclear whether magnolol induces apoptosis of lung carcinoma cells. In this study, magnolol inhibited cell growth, increased lactate dehydrogenase release, and modulated cell cycle in human lung carcinoma A549 cells. Magnolol induced the activation of caspase-3 and cleavage of Poly-(ADP)-ribose polymerase, and decreased the expression level of nuclear factor-κB/*Rel A* in the nucleus. In addition, magnolol inhibited basic fibroblast growth factor-induced proliferation and capillary tube formation of human umbilical vein endothelial cells. These data indicate that magnolol is a potential candidate for treating of human lung carcinoma.

Key words: Magnolol, Apoptosis, Lung carcinoma cells, Caspase-3, Nuclear factor-κB, Angiogenesis

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INTRODUCTION

Lung cancer is a major cause of mortality among Americans, resulting in 159,390 deaths in 2009 and with an estimated 219,440 new cases diagnosed in the same year (Jemal et al., 2009). Lung cancer is a social problem in Korea because of its effect on survival and death rates. According to the Korean Statistical Information Service (KOSIS), the diagnosis of lung cancer in Korea was ranked third in cancer diagnosis behind stomach and colorectal cancers in both 2002 and 2005. The survival rate of patients with stomach cancer was approximately 50% for 5 years, and the survival rates of patients with colorectal and lung cancers were comparatively low for the same period. This statistical data for cancer survival rates in Korea indicates that colorectal and lung cancers are difficult to treat and cure, despite advanced treatments. In particular, lung cancer remains a highly lethal disease because of its continuously high mortality rates and low survival rate.

The two major types of lung cancer are small cell lung cancer (SCLC) and non-SCLC (NSCLC); NSCLC accounts for approximately 85% of all lung cancer cases (Sher et al., 2008). In spite of advances in chemotherapy, the average 5-year survival rate of patients with advanced NSCLC continues to be extremely low (Koshimune et al., 2007). The main curative therapies for lung cancer are surgery and radiation, but only about 20% of tumors are suitable for these treatments, and success is typically only possible with early diagnosis. For advanced tumors, chemotherapy is the treatment of choice. Although chemotherapy, drugs are effective, they are associated with severe adverse side-effects and drug resistance (Kelloff, 2000), and new therapeutic options are needed. Therefore, it is critical to develop novel and effective therapeutic agents that can inhibit the growth of human NSCLC cells.

Magnolol is a phenolic component isolated from the root and bark of *Magnolia officinalis*. It has been used in traditional medicine for the treatment of various

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ailments due to its muscle relaxant, antioxidative, antiatherosclerosis, anti-inflammatory, antigastric ulcer, antiallergic, antibacterial, antithrombotic, and steroidogenesis-stimulating properties (Wang et al., 2000; Ikeda and Nagase, 2002; Lin et al., 2002; Huang et al., 2004; Chen et al., 2005a, 2005b). Recently, a number of studies have drawn attention to the anticancer properties of magnolol. Previous studies have revealed that magnolol inhibits the proliferation and induces the apoptosis of several cancer cell lines including colon cancer, HepG2 hepatoma, leukemia, fibrosarcoma, melanoma, squamous carcinoma, and thyroid carcinoma cells (Lin et al., 2001, 2002; Yang et al., 2003; Zhong et al., 2003; Huang et al., 2007). However, the potential anticancer properties of magnolol against NSCLC are not well-known. In this study, we evaluated the effect of magnolol on the NSCLC A549 cells.

MATERIALS AND METHODS

Materials

Magnolol was purchased from Wako Pure Chemical Industries, Ltd. Fetal bovine serum (FBS), and Roswell Park Memorial Institute (RPMI) 1640 were purchased from GIBCO BRL. Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other reagents were purchased from Sigma Chemical. Procaspase-3, Poly-(ADP)-ribose polymerase (PARP), and nuclear factor-kappaB (NF-κB) p65 antibodies were obtained from Santa Cruz Biotechnology, Inc.

Cell culture

A549 cells (NSCLC, human lung cancer, ATCC Number, CCL-185) were cultured in RPMI 1640 supplemented with 10% FBS. Human umbilical vein endothelial cells (HUVECs) were provided from Innopharmascreen Inc. and cultured in a complete M199 medium (Invitrogen). The cells front passages 3 to 6 were used. A549 and HUVECs were kept at 37°C in a humidified atmosphere of 5% CO_2.

MTT assay

Cells were seeded on 24-well plates at a density of 1×10^5 cells/well in 500 μL of medium. After 24 h of adhesion, the cells were treated with 1 - 100 μM of magnolol and plates were incubated at 37°C for 24 h. The number of living cells was determined with a MTT assay. MTT was dissolved in DMSO at a concentration of 5 mg/mL. From this stock solution, 10 μL per 100 μL of medium was added to each well, and plates were incubated at 37°C for 4 h. Treatment of living cells with MTT produces a dark blue formazan product, whereas no such staining is observed in dead cells. The dark blue crystals that are the metabolized product of MTT were extracted by DMSO. Absorbance at 540 nm was determined and used for the measurement of the proportion of surviving cells.

Cytotoxicity assay

Cytotoxicity induced by different concentrations of magnolol was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. Cells were seeded on 24-well plates at a density of 1×10^5 cells/well in 500 μL of medium. After 24 h of adhesion, the cells were treated 10, 50, and 100 μM of magnolol, and plates were incubated at 37°C for 24 h. Aliquots of supernatant medium and warm reagent of Cytotoxicity Assay Kit (Promega) were mixed in a new 96-well plate. After 30 min, absorbance was measured at a wavelength of 490 nm on a plate reader. Cytotoxicity is presented as percentage of control values.

Morphological observation

Cells were seeded on 24-well plates at a density of 1×10^5 cells/well in 500 μL of medium. After 24 h of adhesion, the cells were treated 10 or 50 μM of magnolol and the plates were incubated at 37°C for 24 h. The morphological changes of the cells were observed with an inverted microscope.

Flow cytometric analysis

Cell cycle pattern was analyzed by the DNA content using propidium iodide (PI) staining method. Briefly, cells were seeded on 6 cm dishes at a density of 1×10^6 cells/well in 2 mL of medium. After 24 h of adhesion, the cells were treated 10 and 50 μM of magnolol, and dishes were incubated at 37°C for 24 h. Cells were washed with cold phosphate buffered saline (PBS), trypsinized, and washed twice again with ice-cold PBS by centrifugation at 500 g for 8 min. After an overnight incubation with 70% methanol at −20°C, the cells were washed again with PBS. Then, final cell pellets were resuspended in 1.0 mL of PI solution (50 μg of PI, 4 mM of sodium citrate, 1 mg/mL of RNase A and 1% of triton X-100 per 1 mL of second distilled water) by gently vortexing. Cells (3×10^5) were incubated for 30 min in the dark, and then analyzed with FACS Vantage SE (Becton Dickinson) using CellQuest Software.

Measurement of caspase-3 activity

The enzymatic activity of caspase-3 was assayed using a caspase colorimetric assay kit (R&D Systems) according to the manufacturer's protocol. Cells were

seeded on 6 cm dishes at a density of 1×10^6 cells/well in 2 mL of medium. After 24 h of adhesion, the cells were treated with 10 or 50 μM of magnolol, and the dishes were incubated at 37°C for 24 h. The cells were lysed in lysis buffer on ice for 15 min. The lysed cells were centrifuged at 2,500 g for 5 min, and protein was incubated with 50 μL of reaction buffer and 5 μL of caspase-3 substrate (DEVD-pNA) at 37°C. After 2 h, the absorbance was measured at a wavelength of 405 nm with a plate reader (Molecular Devices Corp.). Equal amounts of total protein were quantified with a bicinchoninic acid (BCA) protein assay.

Preparation of nuclear extract

After cell activation, cells were washed with ice-cold PBS and resuspended in 60 μL of buffer A (10 mM Hepes/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT and 0.5 mM PMSF, pH 7.9). The cells were allowed to swell on ice for 15 min, lysed gently with 2.5 μL of 10% Nonide P (NP)-40 and centrifuged at 2,000 g for 10 min at 4°C. The supernatant was collected and used as cytoplasmic extract. The nuclei pellet was resuspended in 40 μL of buffer B (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT and 0.5 mM PMSF, pH 7.9), left on ice for 20 min and inverted. The nuclear debris was then spun down at 15,000 g for 15 min. The supernatant (nuclear extract) was collected, frozen in liquid nitrogen and stored at −70°C until ready for analysis.

Western blot analysis

To detect specific protein, cell extracts were prepared with a detergent lysis procedure. Cells were seeded on 6 cm dishes at a density of 1×10^6 cells/well in 2 mL of medium. After 24 h of adhesion, the cells were treated with 10 or 50 μM of magnolol, and the dishes were incubated at 37°C for 24 h. The cells were harvested, washed once with PBS, and resuspended in lysis buffer. Samples were vortexed for lysis for a few seconds every 15 min at 4°C for 1 h and then centrifuged at 15,000 g for 10 min at 4°C. The samples were heated to 95°C for 5 min, cooled on ice, then centrifuged at 3,500 g for 10 min. Total proteins (30 mg) were loaded and separated on SDS-polyacrylamide gels. After electrotransferring onto nitrocellulose membrane (Amersham Pharmacia Biotech) at 4°C, the membrane was blocked with 5% nonfat dry milk in PBS containing 0.05% tween 20 (PBST) for 1 h at room temperature. After slightly washing with PBST, the membrane was probed with various primary antibodies against procaspase-3, PARP, and NF-κB p65 for 12 h at 4°C, then washed three times with PBST. Horseradish peroxidase-conjugated secondary antibodies were used at 1:4,000 dilutions for 2 h at room temperature. The proteins were visualized with enhanced chemiluminescence procedures (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Transient transfection and luciferase assay

For transfection, we seeded A549 cells (1×10^7) in a 100 mm culture dish, and then used Lipofectamine™ 2000 (Invitrogen) to transiently transfect pNF-κB-luciferase (LUC) and pSV40-LUC reporter gene constructs the cells. After 48 h, the transfected cells were seeded on 6 cm dishes at a density of 1×10^6 cells/well in 2 mL of medium. After 24 h of adhesion, the cells were treated with 10 or 50 μM of magnolol, and the dishes were incubated at 37°C for 24 h. The cells were harvested and washed in cold PBS before lysing in a 500 μL lysis buffer (Dual-Luciferase® Reporter Assay System; Promega Corporation). After vortex mixing and centrifugation at 12,000 g for 3 min at 4°C, we stored the supernatant at −70°C until required for the luciferase assay. We mixed 20 μL of cell extract and 100 μL of luciferase assay reagent at room temperature. To measure the luciferase activity, we used a luminometer (1420 luminescence counter, Perkin Elmer) according to the manufacturer's instructions. All transfection experiments were performed three times and received similar results. The relative luciferase activity was defined as the ratio of *firefly* luciferase activity to *renilla* luciferase activity.

In vitro capillary tube formation

Capillary tube formation of HUVECs was performed. Twenty four-well culture plates were coated with 250 μL of Matrigel and allowed to solidify at 37°C for 30 min. HUVEC (2×10^6 cells/mL) in 100 μL, which included extracts and basic fibroblast growth factor (bFGF), or a fresh media as a negative control, were added to three wells and incubated. After 18 h, the capillary tubes were observed under a light microscope (Original magnification × 400).

Statistical analysis

The experiments shown are a summary of the data from at least-three experiments and are presented as the mean ± S.D. Statistical evaluation of the results was performed by ANOVA with Tukey post hoc test. The results were considered significant at a value of $^{***}p < 0.001$, $^{**}p < 0.01$, or $^{*}p < 0.05$.

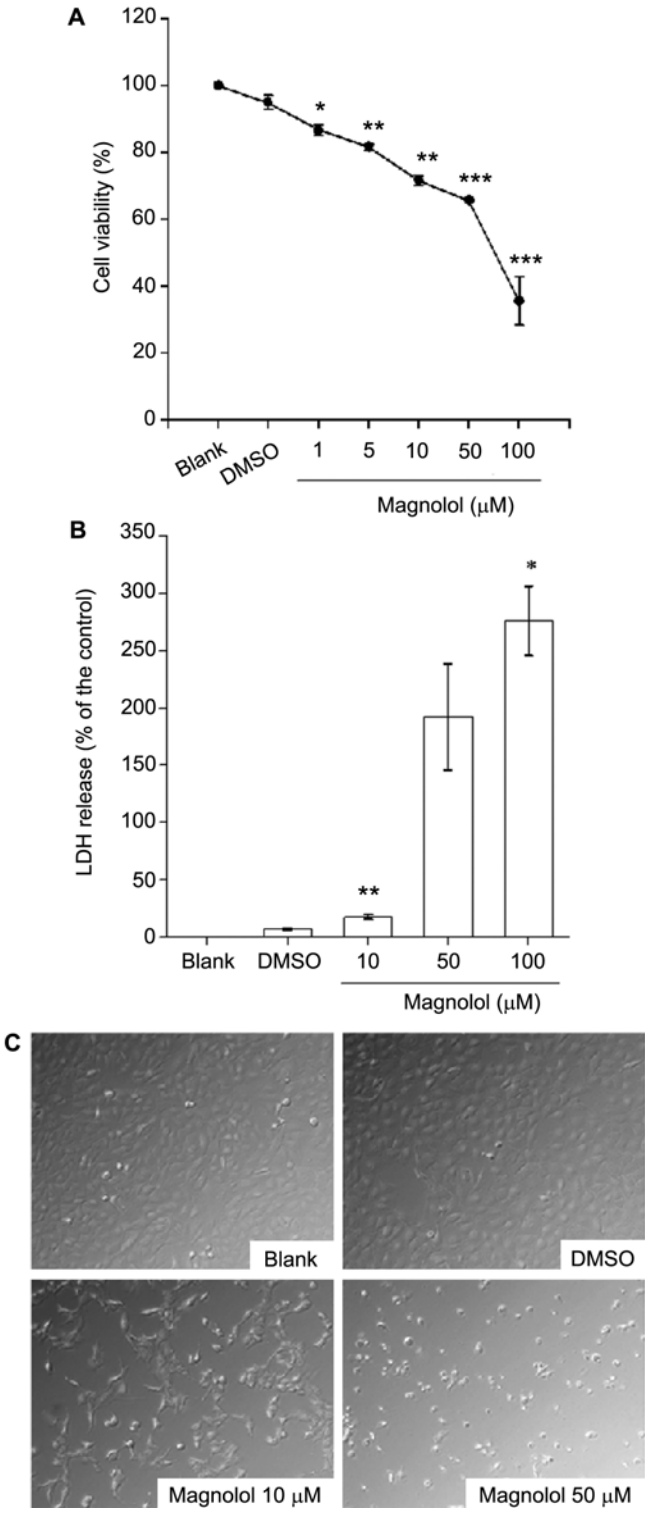

Fig. 1. Magnolol induced cytotoxicity in A549 cells. A549 cells were treated with 1, 5, 10, 50, or 100 μM of magnolol, and 0.1% DMSO as a control (**A**) for 24 h, and cell viability was assessed with a MTT assay. LDH release was also evaluated in A549 cells (**B**). Morphological changes were observed using a microscope (magnification, 200×, **C**). All data are expressed as mean ± S.D. of triplicates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs* blank.

RESULTS

Magnolol induced cytotoxicity in A549 cells

In order to investigate whether magnolol treatments affect cell viability, A549 cells were treated with various concentrations of magnolol for 24 h. As shown in Fig. 1A, treatments with magnolol induced cell death of A549 cells. As shown by Fig. 1B, magnolol also significantly induced LDH release in a dose-dependent manner ($p < 0.05$). Fig. 1C shows microscopic images of the morphological changes in cells treated with magnolol for 24 h. The cells became elongated, there were fewer of them, and they were more disorganized than the untreated cells. The extent of the changes in cell shape and density depended on magnolol concentration.

Magnolol induced high accumulation of sub-G1 phase

The inhibition of cell growth could be due to apoptosis mediated by cell cycle arrest. Since magnolol significantly induced cell death in A549 cells (Fig. 1), we examined whether magnolol affected their cell cycle. As shown in Fig. 2, magnolol treatments increased the population of the sub-G_1 phase when compared with the control.

Magnolol induced activation of caspase-3 and cleavage of PARP

Caspase-3 is known to be an important mediator of apoptosis and to contribute to overall apoptotic morphology by cleaving various cellular substrates (Zhong et al., 2003). Since magnolol treatment induced apoptosis in A549 cells, we investigated whether this phenomenon was the result of the regulation of caspase-3 activation and PARP cleavage. Caspase-3 was significantly activated following treatment with 10 or 50 μM of magnolol (Fig. 3A). Additionally, caspase-3 activation and PARP cleavage were also evaluated using a Western blot analysis. As shown in Fig. 3B, magnolol treatment increased cleavage of pro-caspase-3 and PARP.

Magnolol suppressed NF-κB activation

Kao et al. reported that magnolol creates cytotoxicity by down-regulating NF-κB-dependent antiapoptotic signaling (Kao et al., 2010). To investigate the regulatory effect of magnolol on NF-κB activation in A549 cells, we performed a Western blot analysis of NF-κB/*Rel A* in the nucleus of A549 cells. The expression level of NF-κB/*Rel A* in the nucleus was decreased by treatments of magnolol (10 or 50 μM) (Fig. 4A). To confirm the inhibitory effect of magnolol

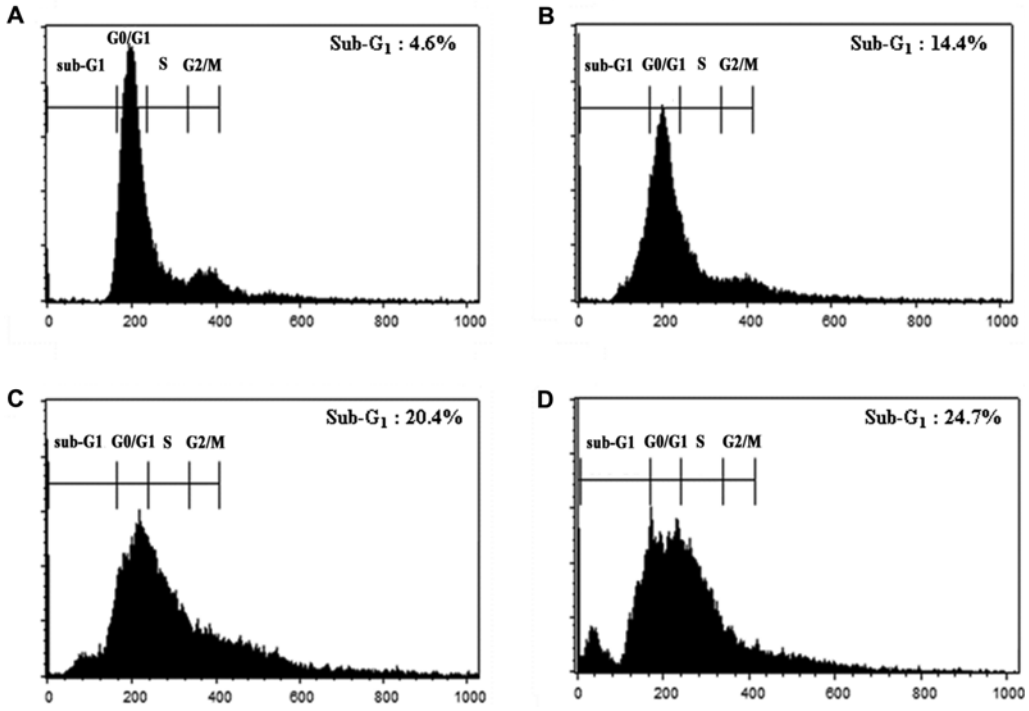

Fig. 2. Magnolol induced high accumulation of sub-G_1 phase. A549 cells were treated with no magnolol (**A**), DMSO (**B**), 10 μM (**C**) or 50 μM (**D**) magnolol for 24 h. At the time indicated, cells were stained with PI, and DNA contents were analyzed with flow cytometry.

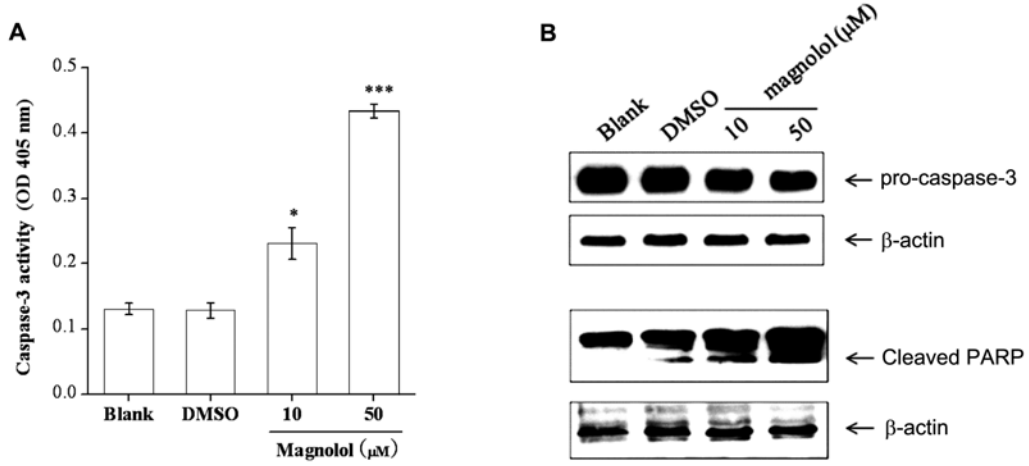

Fig. 3. Magnolol induced activation of caspase-3 and cleavage of PARP. A549 cells were treated with 10 or 50 μM of magnolol for 24 h. The cells were assayed for caspase activity using a caspase colorimetric assay kit (**A**). $*p < 0.05$, $***p < 0.001$ *vs* blank. In parallel, Western blot analysis of procaspase-3 and PARP was performed (**B**). β-actin expression level did not change by any treatment in cell extract. Each Western blot analysis is representative of three independent experiments.

on NF-κB activation, we examined the effect of magnolol in a NF-κB-dependent gene reporter assay. Plasmid NF-κB-luciferase and pSV40-luciferase reporter gene constructs were transiently cotransfected into A549 cells, which was stimulated by magnolol. As shown in Fig. 4B, magnolol significantly reduced luciferase activity in a dose-dependent manner.

Magnolol inhibited bFGF-induced proliferation and angiogenesis in HUVECs

Along with facilitating tumor growth, angiogenesis also enables tumor cells to spread through the

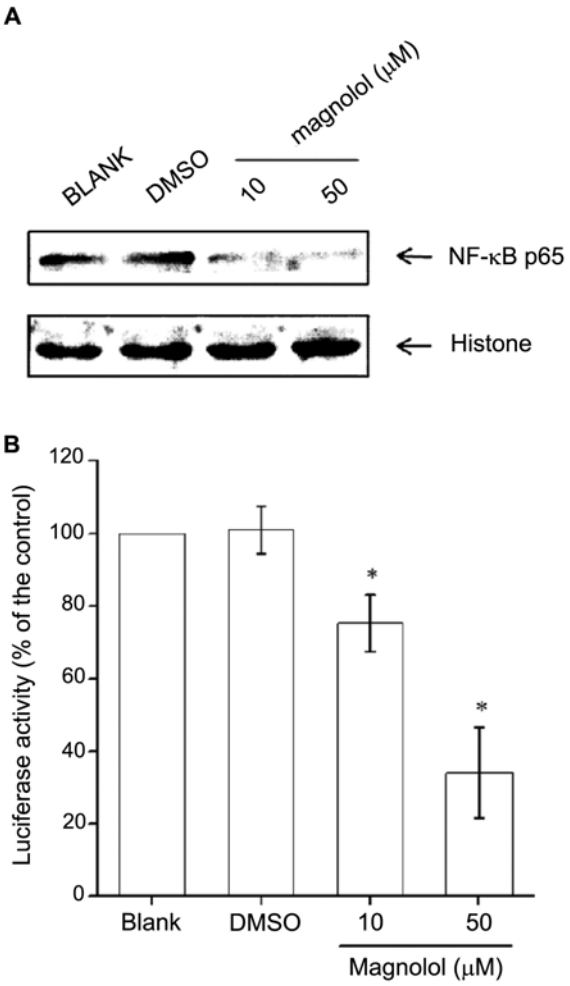

Fig. 4. Magnolol suppressed NF-κB activation. A549 cells were treated with 10 or 50 μM of magnolol for 24 h, and nuclear protein was prepared and analyzed for NF-κB with a Western blotting as described in the methods (**A**). Histone expression level in the nuclear extract did not change with any treatment. The NF-κB activity was assessed with a luciferase assay (**B**). All data are expressed as mean ± S.D. of triplicates. *$p < 0.05$ *vs* blank.

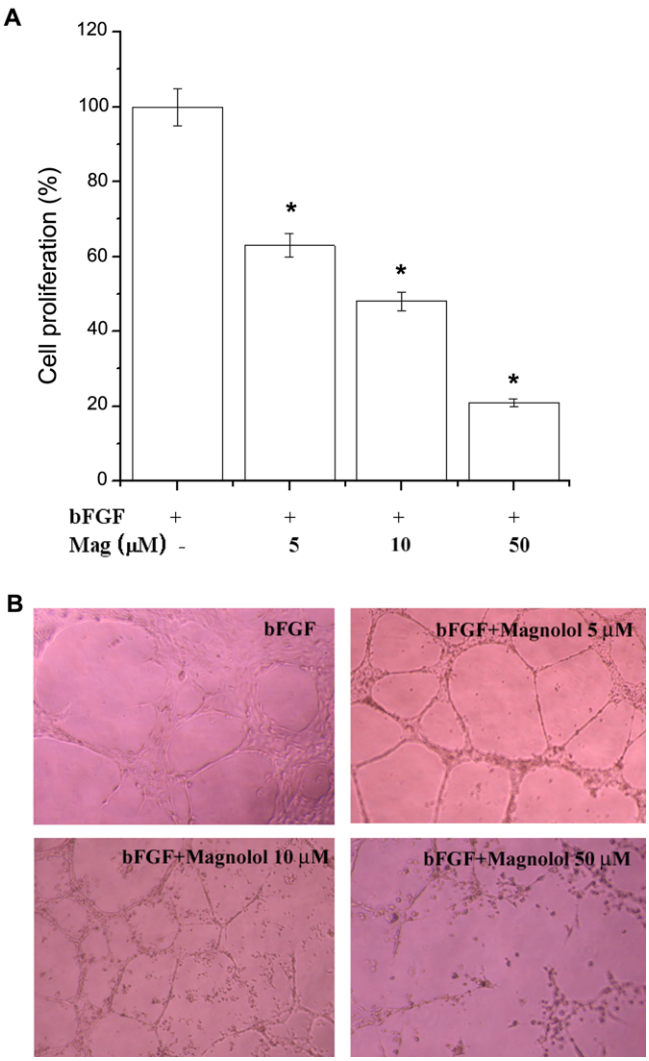

Fig. 5. Magnolol inhibited bFGF-induced angiogenesis in HUVECs**.** HUVECs were incubated with magnolol (5, 10, or 50 μM) for 72 h in the presence of bFGF. After incubation, unbound cells were removed with PBS and incubated with a MTT solution. Absorbance was measured at 540 nm using an ELISA reader (**A**). HUVECs were seeded on Matrigel in 24-well plates in serum-free media containing magnolol in the presence of bFGF (5 ng/mL), or bFGF alone. After 18 h, the capillary tubes were observed using a light microscope. Results are representative of three independent experiments. (Original magnification × 400). *$p < 0.01$ *vs* bFGF. Mag, magnolol.

bloodstream to distant sites. Tumors that grow beyond a certain size trigger angiogenesis by producing an angiogenic molecule, such as bFGF (Gordon et al., 2010). To investigate the biological activities of magnolol, we performed a MTT assay and a tube formation assay using HUVECs. Magnolol markedly inhibited the proliferation of HUVECs compared with the bFGF-treated control group in a dose-dependent manner (Fig. 5A). Next, to examine the effect of magnolol on the differentiation of endothelial cells, HUVECs were suspended in growth factor-reduced Matrigel and then treated with 5, 10, or 50 μM of magnolol in 10 ng/mL of bFGF. As shown in Fig. 5B, 5, 10, or 50 μM of magnolol suppressed bFGF-induced angiogenesis in HUVECs. In particular, treatment with 50 μM of magnolol exhibited the greatest inhibition effect against cell alignments and tubular structure formations.

DISCUSSION

Magnolol has been found to prevent mouse skin tumor formation (Konoshima et al., 1991), human leukemic HL-60 cells growth (Hirano et al., 1994), and human fibrosarcoma HT-1080 invasion (Nagase et al., 2001). Li et al. reported that magnolol induced human NSCLC H460 cells death by autophagy not apoptotic pathways (Li et al., 2007a). Li et al. also reported that magnolol contributed to H460 cells survival by down-regulating p53 phosphorylation and up-regulating Akt phosphorylation (Li et al., 2007b). Therefore, we understand that effects of magnolol are different according to cell type and condition. Up to now, it has been unclear whether magnolol induces apoptosis in lung carcinoma cells. In this study, we demonstrated that magnolol suppressed cell growth by inducing the sub-G_1 cell cycle accumulation through regulation of caspase-3 and NF-κB pathway in human lung cancer carcinoma A549 cells.

Apoptosis is important not only during development and tissue homeostasis, but also in the pathogenesis of a variety of human disorders (Woodle and Kulkarni, 1998; Dove, 2001). The derangements of apoptosis can have deleterious consequences as exemplified by several human diseases, including acquired immunodeficiency syndrome, neurodegenerative disorders, and cancer (Thompson, 1995). In this study, we observed that treatment with magnolol significantly inhibited A549 cell viability in a dose-dependent manner. The morphology of the cells also demonstrated that magnolol induced their death. We also confirmed that magnolol increased the sub-G_1 portion of the cell cycle in A549 cells. These results indicate that magnolol is associated with the induction of apoptosis in A549 cells, shown by a loss of cell viability and an accumulation of cells in the sub-G_1 phase.

In mammalian cells, there are two major caspase activation pathways: the extrinsic and intrinsic pathways (Thompson, 1995). In the extrinsic pathway, binding of the death receptors causes the activation of caspase-8, which is an initiator caspase. In the intrinsic pathway, various forms of cellular stress cause mitochondrial alterations leading to mitochondrial membrane depolarization and the release of cytochrome c (Cyto c). In the cytosol, Cyto c binds to and activates Apaf-1, which then activates pro-caspase-9. Active caspase-9 directly cleaves and activates the effector protease, caspase-3. Activation of caspase-3 is regarded as a primary mechanism of apoptosis (Cohen, 1997; Nagata, 1997). Activated caspase-3 induced PARP fragmentation (Ashkenazi and Dixit, 1998; Hengartner, 2000). Detection of PARP cleavage fragment has been shown to be a hallmark of apoptosis. Other study reported that magnolol induced apoptosis through the activation of caspase-3 in HepG2 (Lin et al., 2002). However, the effect of magnolol on caspase pathway in A549 cells is not elucidated. Although the apoptotic pathway induced by anticancer agents is controversial, some studies strongly suggest that caspases play a key role in anticancer agents. For example, Nimesulide, a selective COX-2 inhibitor, induced apoptosis through caspase-3 and caspase-8 in A549 cells (Kim et al., 2009). PAC-1, a small molecule, is also apoptosis-inducible agent through caspase-3 both *in vitro* and *in vivo* cancer models (Putt et al., 2006). While other studies suggest that NV-128, a novel isoflavone derivative, and capsaicin induced the apoptosis through caspase-independent pathway (Alvero et al., 2009; Chou et al., 2009). This apparently conflicting data suggests that the pathways of anticancer agents-induced apoptosis differ according to the cancer cell type or the exposure (cell treatment) conditions. In this study, we showed that magnolol induced caspase-3 activation and PARP cleavage. These results indicate that treatment with magnolol induces apoptotic death in A549 cells through the activation of capase-3 and subsequent cleavage of PARP. Therefore, we believe that the apoptotic mechanism of magnolol in lung cancer might occur at least partially through a caspase-dependent pathway.

Recent evidence indicates that NF-κB signaling pathways are significantly involved in tumor development (Nakshatri et al., 1997). Since anticancer treatments result in the inhibition of NF-κB transcriptional activity, the inhibition of NF-κB in conjunction with chemotherapy strongly enhances its cytotoxic effects. NF-κB may play an important role in causing drug resistance, and so the inactivation of NF-κB by therapeutics is intended as a new strategy for to eliminating cancerous cells with apoptosis (Herr and Debatin, 2001). In lung cancer, the activation of NF-κB has been associated with several aspects of tumorigenesis such as tumor cell growth, antiapoptosis, metastasis, angiogenesis, resistance against chemotherapeutics, and tumor promotion (Nakshatri et al., 1997). Therefore, the activation of NF-κB may create favorable conditions for lung cancer cell growth. This suggests that the NF-κB pathway is a potential therapeutic target for lung cancer. In our study, we found that magnolol decreased NF-κB activation, and so it might be a potential therapeutic treatment for lung cancer.

The growth of tumors is dependent on an adequate supply of oxygen and nutrients, which are supplied to the tumor by blood vessels. Tumor progression is

dependent on tumor angiogenesis i.e. the growth of new blood vessels into tumors (Reynolds, 2010). Angiogenesis is a complicated process that includes the proliferation and migration of endothelial cells, which is controlled by a delicate balance between angiogenic inducers and angiogenic inhibitors (Fan et al., 1995; Bussolino et al., 1997). The fact that the growth and spread of tumors is dependent on angiogenesis has led to investigation into the role of antiangiogenic agents in therapeutic strategies for thoracic tumors such as NSCLC or mesotheliomas. Hu et al. reported that BPR0C261 is a novel orally-administered active antitumor agent with antimitotic and antiangiogenic properties (Hu et al., 2011). In this study, we demonstrated that magnolol suppressed bFGF-induced angiogenesis in HUVECs. In addition, magnolol induced cell death of HUVECs. These results indicate that magnolol not only induces apoptosis but also limits angiogenic progress in cancer models. In other words, magnolol has potential as a therapeutic cancer agent with apoptotic and antiangiogenic properties. In this study, we investigated the effect of magnolol on tube formation of HUVECs with concentrations displaying cytotoxicity in A549 cells. Further study is necessary to understand the effect of low concentrations of magnolol, those without cytotoxicity on tube formation.

In conclusion, magnolol induced apoptosis through activation of caspase-3 and the suppression of NF-κB activation. Magnolol also inhibited proliferation and tube formation in HUVECs. Combined, these findings indicate that magnolol may be a potent anticancer treatment that could be used in combination with conventional chemotherapeutic treatments for human lung cancer carcinoma.

ACKNOWLEDGEMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (No. 2005-0049404).

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