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Journal of Nutritional Biochemistry 23 (2012) 706–715

Journal of Nutritional Biochemistry

4-*O*-methylhonokiol inhibits colon tumor growth via p21-mediated suppression of NF-κB activity☆

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Received 30 October 2010; received in revised form 24 February 2011; accepted 14 March 2011

Abstract

Biphenolic components in the *Magnolia* family have shown several pharmacological activities such as antitumor effects. This study investigated the effects of 4-*O*-methylhonokiol (MH), a constituent of *Magnolia officinalis*, on human colon cancer cell growth and its action mechanism. 4-*O*-methylhonokiol (0–30 μM) decreased constitutive activated nuclear factor (NF)-κB DNA binding activity and inhibited growth of human colon (SW620 and HCT116) cancer cells. It also caused G_0–G_1 phase cell cycle arrest followed by an induction of apoptotic cell death. However, knockdown with small interfering RNA (siRNA) of p21 or transfection with cyclin D1/Cdk4 binding site-mutated p21 abrogated MH-induced cell growth inhibition, inhibition of NF-κB activity as well as expression of cyclin D1 and Cdk4. Conversely, inhibition of NF-κB with specific inhibitor or siRNA augmented MH-induced apoptotic cell death. 4-*O*-methylhonokiol inhibited tumor growth, NF-κB activity and expression of antiapoptotic proteins; however, it increased the expression of apoptotic proteins as well as p21 in xenograft nude mice bearing SW620 cancer cells. The present study reveals that MH causes p21-mediated human colon cancer cell growth inhibition through suppression of NF-κB and indicates that this compound by itself or in combination with other anticancer agents could be useful for the treatment of cancer.

Keywords: 4-*O*-methylhonokiol; NF-κB; p21; Cancer cells; Growth inhibition

1. Introduction

The root and stem bark of *Magnolia officinalis* have been used for oriental medicine since this medicinal herb and its bioactive constituents such as honokiol, obovatol and magnolol exhibit a variety of biological effects including antitumor, antimicrobial, anti-inflammatory, antithrombotic and anxiolytic effects [1–3]. Several studies have shown that honokiol has antiangiogenic, antiproliferative and anti-invasive activities in colon and prostate cancer cells as well as other cancer cells such as lung, breast and ovarian cancer cells [3–7]. Magnolol has also been shown to induce apoptosis, suppress proliferation of cancer cells and inhibit tumor metastasis in a variety of cancer cells including colon and prostate cancer cells [8,9]. We recently also found that obovatol inhibit colon and prostate cancer cell growth [10].

The nuclear factor (NF)-κB is a transcription factor regulating various genes involved in the production of inflammatory cytokines, chemokines, cell adhesion molecules and growth factors [11]. It mediates tumor promotion and progression, angiogenesis, metastasis of cancer cells and resistance against chemotherapeutics through increases of the expression of genes participating in cancer development [12,13]. It also regulates expression of a number of antiapoptotic proteins and apoptotic genes as well as proliferation genes [14]. Interestingly, NF-κB is constitutively activated in a variety of solid tumors including animal and human colon tumor [15,16]. Constitutive activation of NF-κB has been found to up-regulate antiapoptotic genes expression and therefore disrupt the balance between apoptosis and proliferation, leading to cancer cell growth [17]. Thus, inhibition of NF-κB has been viewed as a key regulator of cell growth of various cancer cells [18], and several studies have reported that a variety of natural compounds such as polyphenols, lignans, sesquiterpenes, diterpenes and triterpenoid suppressing NF-κB activity inhibits cancer cell growth [19–21]. We have also demonstrated that suppression of NF-κB by naturally occurring compounds such as inflexinol, thiacremonone and cobrotoxin inhibits colon cancer cell growth [22,23].

Functional inactivation of the p21 pathway has been observed in most human tumors [24,25]. p21 is an important cellular checkpoint

Abbreviations: NF-κB, nuclear factor-κB; EMSA, electromobility shift assay; c-IAP1, cellular inhibitor of apoptosis protein 1; TUNEL, TdT-mediated dUTP nick and labeling; DAPI, 4,6-diamino-2-phenylindole.

☆ Conflicts of interest statement: The authors declare that there are no conflicts of interest.

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doi:10.1016/j.jnutbio.2011.03.013

protein for the inhibition of cell growth through the control of the cyclin-CDKs activities in human cancer cells [26]. Nuclear factor-κB is also important in the p21-mediated cell cycle arrest of human breast cancer cells as well as colon cancer cells [27]. Nuclear factor-κB-dependent p21 expression was observed in the resistance of human leukemic cells [28]. Moreover, a recent study demonstrated that aqueous extract of *Magnolia officinalis* showed increased p21 expression and decreased NF-κB on human urinary bladder cancer cells [29]. We recently isolated a novel bioactive compound, 4-*O*-methylhonokiol (MH), from *Magnolia officinalis* and found that it suppresses NF-κB activation in macrophages [30]. Moreover, it has been suggested that the growth inhibitory effect of the bioactive constituents of *Magnolia officinalis*, obovatol, honokiol and magnolol, in cancer cells is associated with the inhibitory ability of these compounds on NF-κB activity [10,29].

Thus, we hypothesized that MH might inhibit growth of human colon cancer cells through inactivation of NF-κB, and its action could be mediated by the p21 induction. Here, we show that MH decreases NF-κB activity and cell growth of colon cancer cell lines via induction of p21.

2. Materials and methods

2.1. Reagents

4-*O*-methylhonokiol (Supplementary Fig. 1A) was isolated form a bark of *Magnolia officinalis* Rehd. et Wils by subsequently extracting with *n*-hexane, ethyl acetate and *n*-BuOH, and then identified by ^{1}H-NMR and ^{13}C-NMR as described elsewhere [31]. 4-*O*-methylhonokiol was supplied by Bioland Ltd. (Chungnam, Korea) as a brown oilish solution of 99.6% purity. Stock solution of MH was diluted in 0.05% dimethyl sulfoxide (DMSO) (final concentration, 50 mM).

2.2. Cell culture

SW620 and HCT116 human colon cancer cells and CCD 112-CoN human colon normal cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The colon cancer cells were grown in RPMI 1640, and the CCD 112-CoN cells were grown in Eagle's minimum essential medium with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3. Cell growth assay

Cells (5×10^4 cells/well) were plated onto 24-well plates. The cell growth inhibitory effect was evaluated in the cells treated with MH (0–30 μM) for 0–72 h using excluded trypan blue assay.

2.4. Transfection and assay of luciferase activity

Cells (1×10^5 cells/well) were plated in 24-well plates and transiently transfected with pNF-κB-Luc plasmid (5× NF-κB; Stratagene, La Jolla, CA, USA) using a mixture of plasmid and Lipofectamine PLUS in OPTI-MEN according to manufacturer's specification (Invitrogen, Carlsbad, CA, USA). The transfected cells were treated with MH in the absence or presence of tumor necrosis factor (TNF)-α (10 ng/ml) for 8 h. Luciferase activity was measured by using the luciferase assay kit (Promega, Madison, WI, USA).

2.5. Electromobility shift assay

Electromobility shift assay (EMSA) was performed according to the manufacturer's recommendations (Promega, Madison, WI, USA) as described elsewhere [22].

2.6. Western blot analysis

Protein (40 μg) from cultured cells was separated on a sodium dodecyl sulfate (SDS)/12%-polyacrylamide gel and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The membranes were then immunoblotted with primary specific antibodies: rabbit polyclonal antibodies for p65, bax, bcl-2, GSK-3β (1:500 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), caspase-3, caspase-9, pp53, survivin, cellular inhibitor of apoptosis protein 1 (c-IAP1) (1:1000 dilution, Cell Signaling Technology, Inc., Beverly, MA, USA) and c-IAP2 (1:1000 dilution, Abcam Ltd., Cambridge, UK) and mouse monoclonal antibodies for p50, p53, CDK6 (1:500 dilution, Santa Cruz Biotechnology Inc.), CDK4, p21, cyclin D, cyclin E (1:1000 dilution, Medical and Biology Laboratories Co. Ltd., Nagoya, Japan), iNOS and COX-2 (1:1000 dilution, Cayman chemical company, Ann Arbor, MI, USA). The blot was then incubated with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (1:2000 dilution, Santa Cruz Biotechnology Inc.).

2.7. Cell cycle analysis by flow cytometry

Subconfluent cells were treated with MH (0–30 μM) in culture medium for 0–72 h. The cells were harvested by trypsin-EDTA release and fixed in ice-cold 70% ethanol. At least 1–2 h before flow cytometric analysis, cells then were washed twice with ice-cold phosphate-buffered saline (PBS) and incubated with RNase and the DNA intercalating dye propidium iodide. Cell-cycle phase analysis was performed using a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA). Flow cytometric analysis was done with flow cytometry system (FACSCalibur-S System, BD Bioscience). Cells in the G_1, S and G_2–M phases of the cell cycle were determined with Modfit LT (Verity House Software, Topsham, ME, USA).

2.8. Transfection of siRNA and p21 mutant plasmid

Cells were transfected with 100 nM of CDKN1A (p21), NF-κB (p50 or 65) siRNA or nonspecific siRNA (Bioneer Co., Daejon, Korea) and p21 mutant plasmid (CDKN1A p21, obtained from Dr. Anindya Dutta, Department of Biochemistry and Molecular Genetics, University of Virginia School of Medicine, Charlottesville, VA, USA) that has mutated cyclin D1/cdk4 complex binding site (Cy1 site, △17–24) [32] using WelFect-EX plus transfection reagent (WelGENE, Seoul, Korea) prepared in a serum-free culture medium at 37°C. After 6 h, a complete medium was added, and the cells were further cultured for 24 h.

2.9. Detection of apoptosis

Cells (1×10^4 cells/well) were cultured in eight-well chamber slides (BD Biosciences, Bedford, MA, USA) for 24 h. For detection of apoptotic cell death in tumor tissues and cultured cells, TdT-mediated dUTP nick and labeling (TUNEL) assays were performed by using the *in situ* cell death detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's instructions as described elsewhere [22]. The apoptotic index was determined as the number of 4,6-diamino-2-phenylindole (DAPI)-stained TUNEL-positive stained cells divided by the total cell number counted ×100.

2.10. Antitumor activity study in vivo xenograft animal model

Six-week-old male BALB/c athymic nude mice were purchased from Japan SLC (Hamamatsu, Japan). All experiments were approved and carried out according to the *Guideline for the Care and Use of Animals* of the Chungbuk National University Animal Care Committee. SW620 cancer cells were injected subcutaneously (1×10^7 cells/0.1 ml PBS/animal) into the lower right flanks of mice. After 20 days, when the tumors had reached an average volume of 300–400 mm^3 or about 50 mm^3 (for prostate), the tumor-bearing nude mice were intraperitoneally (ip) injected with MH (40 and 80 mg/kg dissolved in 0.1% DMSO) twice per week for 3 weeks. Cisplatin (10 mg/kg, Choongwae Pharma Co., Seoul, Korea) was also intraperitoneally injected once a week as a positive control. The group treated with 0.1% DMSO was designated as the control. The tumor volumes were measured with vernier calipers and calculated by the following formula: $(A\times B^2)/2$, where A is the larger and B is the smaller of the two dimensions.

2.11. Immunohistochemistry

Immunohistochemistry was done as described elsewhere [22] with 5-μm-thick tissue sections using primary mouse PCNA, Ki-67 and p65 (1:200 dilution) antibodies or primary rabbit p50 and cleaved caspase-3 antibody (1:100 dilution) followed by a secondary biotinylated antimouse or rabbit antibody for 30 min. The tissue sections were developed with diaminobenzidine and peroxide, then counterstained with hematoxylin, mounted in Aqua-Mount and evaluated on a light microscopy (Olympus, Tokyo, Japan). A negative control was performed in all cases by omitting the primary antibody. All sections were counterstained with hematoxylin. For quantification, 200 cells at three randomly selected areas were assessed, and the positively stained cells were counted and expressed as percentage of stained cells.

2.12. Data analysis

Data were analyzed using GraphPad Prism 4 software (Version 4.03, GraphPad Software, Inc.). Data were assessed by one-way analysis of variance (ANOVA). If the P value in the ANOVA test was significant, the differences ($P<.05$) between the pair of means were assessed by the Dunnett's test. Data are presented as mean±S.D. from three independent experiments with triplicates.

3. Results

3.1. MH inhibited NF-κB activity and cancer cell growth in human colon cancer cells

Since highly activated NF-κB is an implicated factor in cell survival as well as in the resistance against therapeutics of colon cancer cells, we first determined the inhibitory ability of MH on the DNA binding activity of NF-κB. We found a high-level constitutive activation of the DNA binding activity of NF-κB in the untreated cells. However, treatment of MH for 1 h inhibited the constitutively activated DNA binding activity of NF-κB in a concentration-dependent manner (0–30 μM) in human colon cancer cells (SW620 and HCT116) (Fig. 1A). This DNA binding activity of NF-κB was confirmed by a competition assay using excessive non-(γ-^{32}P) ATP-labeled oligonucleotide of NF-κB consensus and a supershift assay using p50 and p65 antibodies in SW620 cells (Supplementary Fig. 1B). Consistent with the inhibitory effects on the NF-κB DNA binding activity, MH (0–30 μM) concentration dependently inhibited TNF-α-induced NF-κB luciferase activity (Fig. 1B). Confocal microscope analysis further confirmed that the translocation of NF-κB subunits p50 and p65 into the nucleus was also decreased by MH (Fig. 1C). In agreement with the relationship between the inhibition of NF-κB activity and cancer cell growth, MH (0–30 μM) treatment resulted in a significant concentration- and

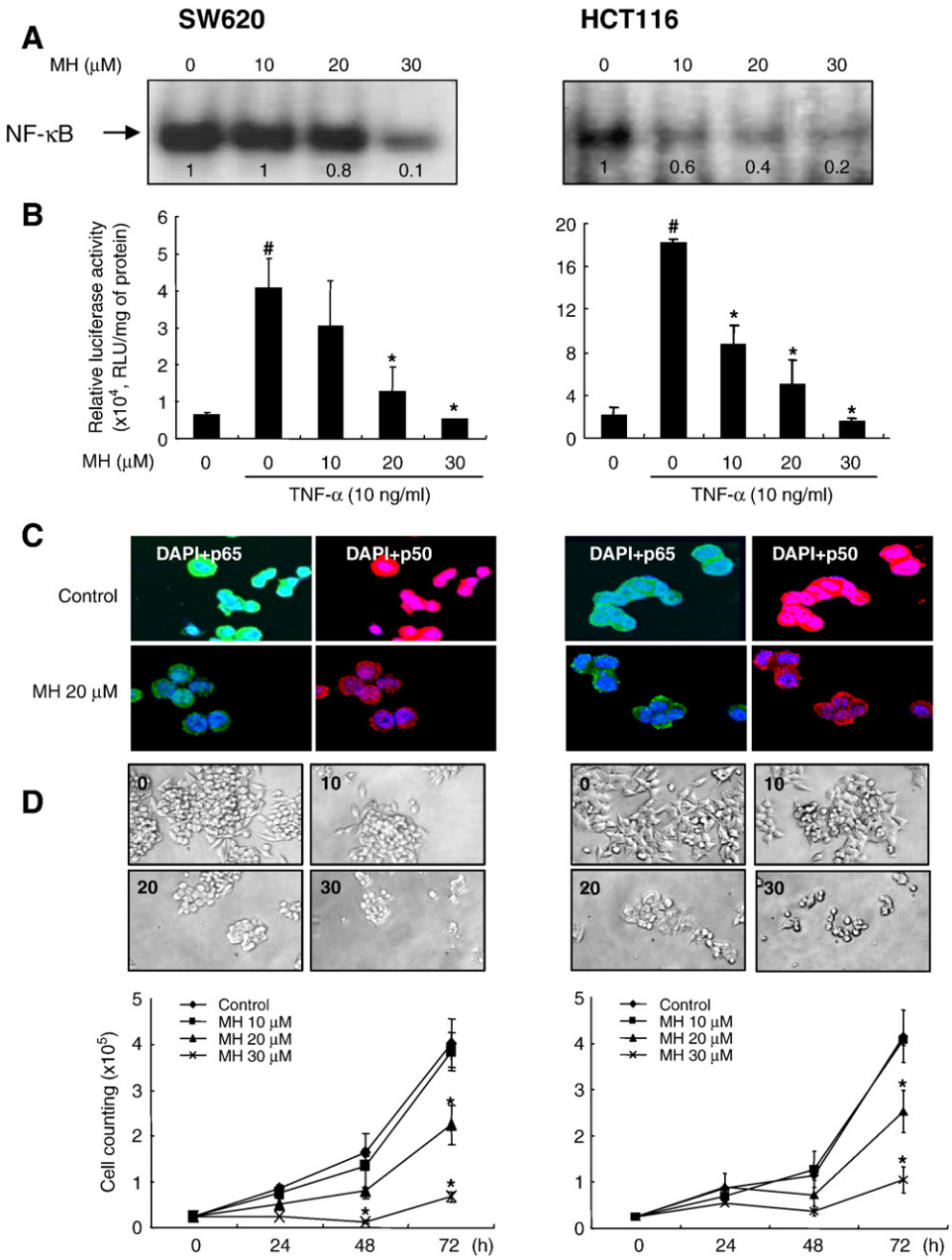

Fig. 1. Effect of MH on activation NF-κB and cancer cell growth. Cells were treated with 0–30 μM MH for 1 h. (A) EMSA and (C) immunofluorescence were performed as described in Materials and methods. *Numbers*, fold activation in relation to the control. (B) Cells were transfected with pNF-κB-Luc plasmid (5× NF-κB) for 6 h and then incubated with complete media containing 0–30 μM MH for 8 h. Luciferase activity was performed as described in Materials and methods. *Columns*, mean of triplicate; *bars*, S.D. (D) Cells were treated with 0–30 μM MH for different times (0–72 h). Cell counting was performed as described in Materials and methods. *Points*, mean of triplicate; *bars*, S.D. $^{*}P<.05$ indicates statistically significant differences from the untreated group. $^{\#}P<.05$ indicates statistically significant differences from the TNF-α-treated group.

time-dependent inhibition of cell growth in colon cancer cells (Fig. 1D). However, MH was not cytotoxic in the normal colon CCD-112 CON cells in the tested concentration (Supplementary Fig. 1C). When we compared cell growth inhibition and NF-κB DNA binding activity by honokiol, magnolol, obovatol and MH, the extent of cell growth inhibition was in agreement with the inhibition of NF-κB with the exception of the effect of obovatol (a much greater effect) in PC-3 cells (Supplementary Fig. 1D).

3.2. MH caused G_0/G_1 phase cell cycle arrest and induced apoptotic cell death

Exposure of cells to MH (0–30 μM) resulted in the enrichment of G_0/G_1 fraction in colon cancer cells in a concentration- and time-dependent manner (Fig. 2A). According to the cell cycle pattern, MH caused a rapid and marked decrease in protein levels of Cdk2, Cdk4, cyclin D1 and cyclin E (Fig. 2B). In consideration of the significance of the Cdk inhibitor p21 in the regulation of G_0/G_1 transition by binding to Cdk/cyclin complexes that phosphorylate Rb and p53, leading to cell cycle progression [33], we determined its effect on protein levels and/or phosphorylation of p21, Rb and p53 by immunoblotting. As shown in Fig. 2B, MH resulted in the increase of p21 and phosphorylation of p53 protein expression (SW620 cells were not detected since these lack or have no p53), but suppressed Rb phosphorylation (Fig. 2B). These results indicated that MH-mediated cell cycle arrest in colon cancer cells was associated with the G_0/G_1 cell cycle regulation through induction of p21. Apoptotic cell numbers were increased to 2%±3%, 10%±5%, 55%±13% and 76%±8% in SW620 cells and 2%±4%, 15%±18%, 45%±5% and 73%±7% in HCT116 cells by 0–30 μM MH, respectively (Fig. 2C), indicating that cell cycle arrest was followed by the induction of apoptosis. The expression of antiapoptotic proteins bcl-2 and the inhibitor of apoptosis protein

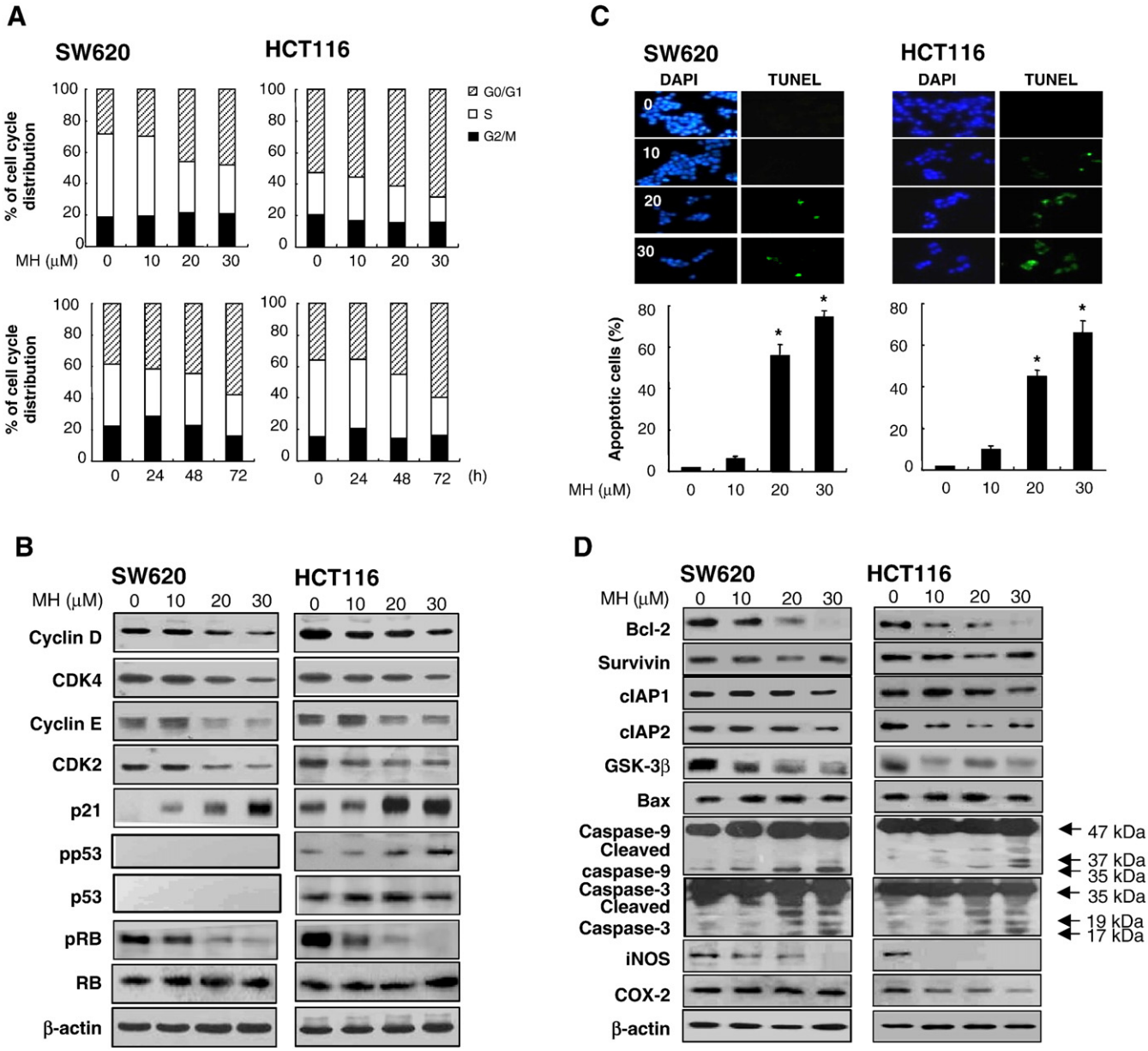

Fig. 2. Effect of MH on cell cycle and apoptosis. (A) Cells were treated with 0–30 μM MH for 72 h or 20 μM MH for 0–72 h. Cell cycle was performed as described in Materials and methods. (A–D) Cells were treated with 0–30 μM MH for 24 h. (A and D) Western blotting and (C) DAPI and TUNEL staining were performed as described in Materials and methods. *Columns*, mean of triplicate; *bars*, S.D. $^*P<.05$ indicates statistically significant differences from the untreated group.

1/2 (c-IAP1/2) as well as GSK-3β and survivin was decreased (Fig. 2D), but the expression of proapoptotic proteins bax, cleaved caspase-3 and cleaved caspase-9 was increased by MH (Fig. 2D). Moreover, the expression of COX-2 and iNOS, which are involved in the tumor promotion of prostate and colon cancer, was significantly decreased by MH (Fig. 2D).

3.3. Knockdown of NF-κB augmented the inhibitory effects of MH on cancer cell growth and apoptotic cell death

To further determine the role of NF-κB inhibition in cancer cell growth, cells were pretreated with siRNA p65 or p50 30 min prior to the treatment of MH (20 μM). Cell growth inhibition by MH was augmented in the SW620 cells transfected with p50 and p65 siRNA or treated with the NF-κB inhibitor phenylarsine oxide (PAO, 0.5 μM) compared to the cells treated with MH alone (Fig. 3A). Apoptotic cell death was also augmented in the p50 and p65 siRNA-transfected or PAO-treated cells (Fig. 3B). The expression of p21, cyclin D1 and Cdk4 was also significantly reduced, but the expression of cleaved caspase-3 was much highly elevated in the p50 and p65 siRNA-transfected or PAO-treated cells (Fig. 3C). These results further confirmed that MH-induced cell growth inhibition of both SW620 cancer cells could be correlated with the inhibition of the NF-κB signal.

3.4. Knockdown of p21 abolished the effects of MH on cancer cell growth inhibition, induction of apoptotic cell death and NF-κB inactivation

The p21 protein regulates G_0/G_1 transition by binding to Cdk/cyclin complexes that phosphorylate Rb and p53, leading to cell cycle progression. Nuclear factor-κB also regulates genes involved in cell proliferation (such as cyclin D1 and COX-2). To further investigate the involvement of p21 in the MH-induced inactivation of NF-κB and thereby inhibition of cell growth, we employed p21 siRNA. p21 siRNA resulted in the abolition of the effect of MH on cancer cell growth inhibition (Fig. 4A) and induction of apoptotic cell death (Fig. 4B). Moreover, p21 siRNA-transfected cells abolished MH-induced inhibition of the cell cycle-regulatory and NF-κB subunit proteins' expression (Fig. 4C). In addition, similar to the abolished effect of p21 siRNA, the cells transfected with mutant p21 (mutation of cyclin D1 and Cdk4 complex binding site of p21) also abolished the inhibitory effects of MH on cell growth, cell cycle-regulatory and NF-κB subunit proteins' expression (Fig. 5A and 5B). These results indicated that NF-κB signal-mediated inhibitory effects of MH on the growth of both SW620 colon cancer cells could be associated with the activation of p21 signal pathway.

3.5. MH inhibited the SW620 and PC3 tumor growth in in vivo xenograft model

In SW620 xenograft studies, MH was administrated ip every day for 4 weeks to mice with tumors ranging from 100 to 300 mm^2 in volume. On day 28, the final tumor weight was recorded. Tumor volumes in mice treated with MH at 40 and 80 mg/kg and cisplatin at 10 mg/kg were 40.9%, 34.1% and 27.3% of control group in SW620 tumor xenografts, respectively. Tumor weight in mice treated with MH at 40 and 80 mg/kg and cisplatin at 10 mg/kg were 45.1%, 25.8% and 9.6% of control group in SW620 tumor xenografts, respectively (Fig. 6A). The immunohistochemical analysis of tumor sections by hematoxylin and eosin and proliferation antigens against PCNA and Ki67 staining revealed that 40 and 80 mg/kg dose-dependently inhibited both tumor cell growths (Fig. 6B). In addition, there was a trend toward decreased intensity of nuclear staining of p65 and p50 in

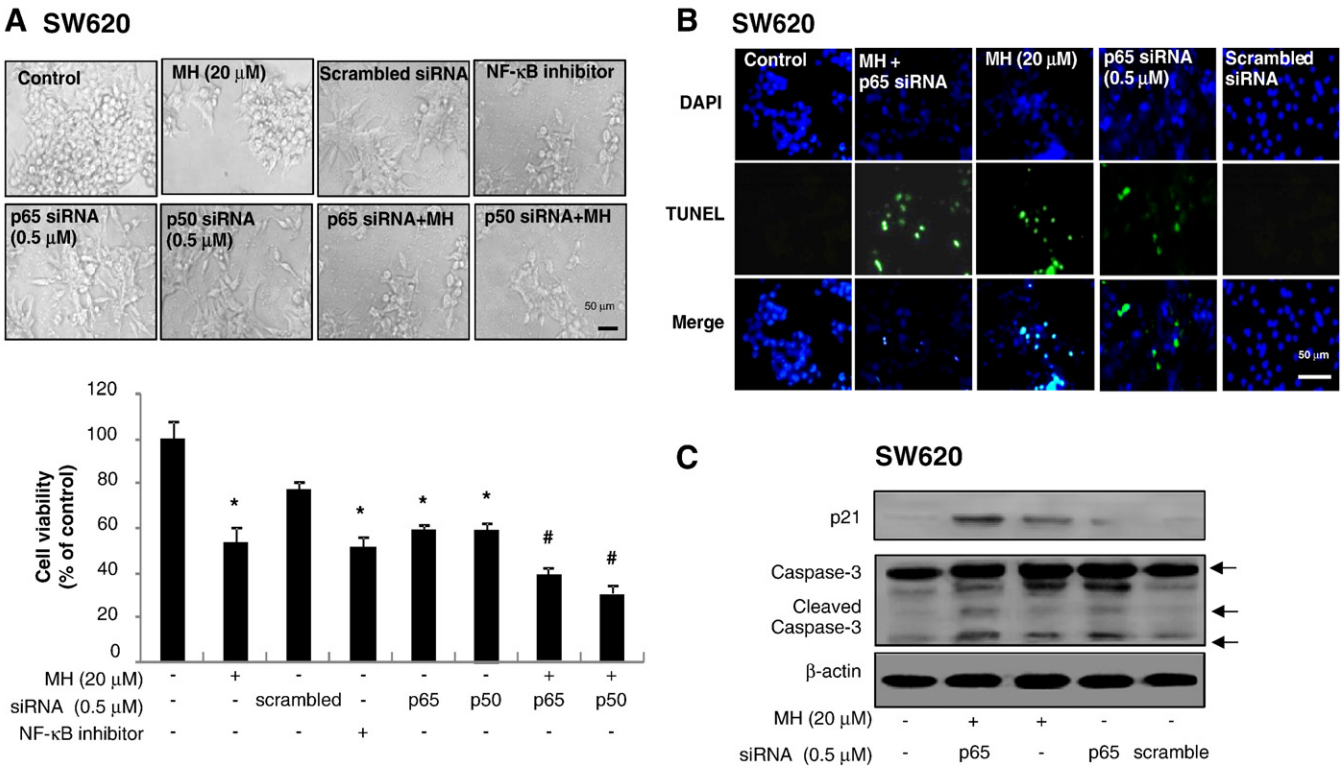

Fig. 3. Augmented effect of NF-κB inhibitor (PAO) or siRNA on the cell viability, apoptosis and related protein expression in MH-treated SW620 cancer cells. The cells were pretreated with 0.5 μM NF-κB inhibitor (PAO) or transfected with NF-κB siRNA for 6 h, grown for 24 h in complete media and treated with 20 μM MH for 24 h. (A) Cell counting, (B) DAPI and TUNEL staining and (C) Western blotting were performed as described in Material and methods. *Columns*, mean of triplicate; *bars*, S.D. $^{*}P<.05$ indicates statistically significant differences from the untreated group. $^{\#}P<.05$ indicates statistically significant differences from the MH- or siRNA-treated group.

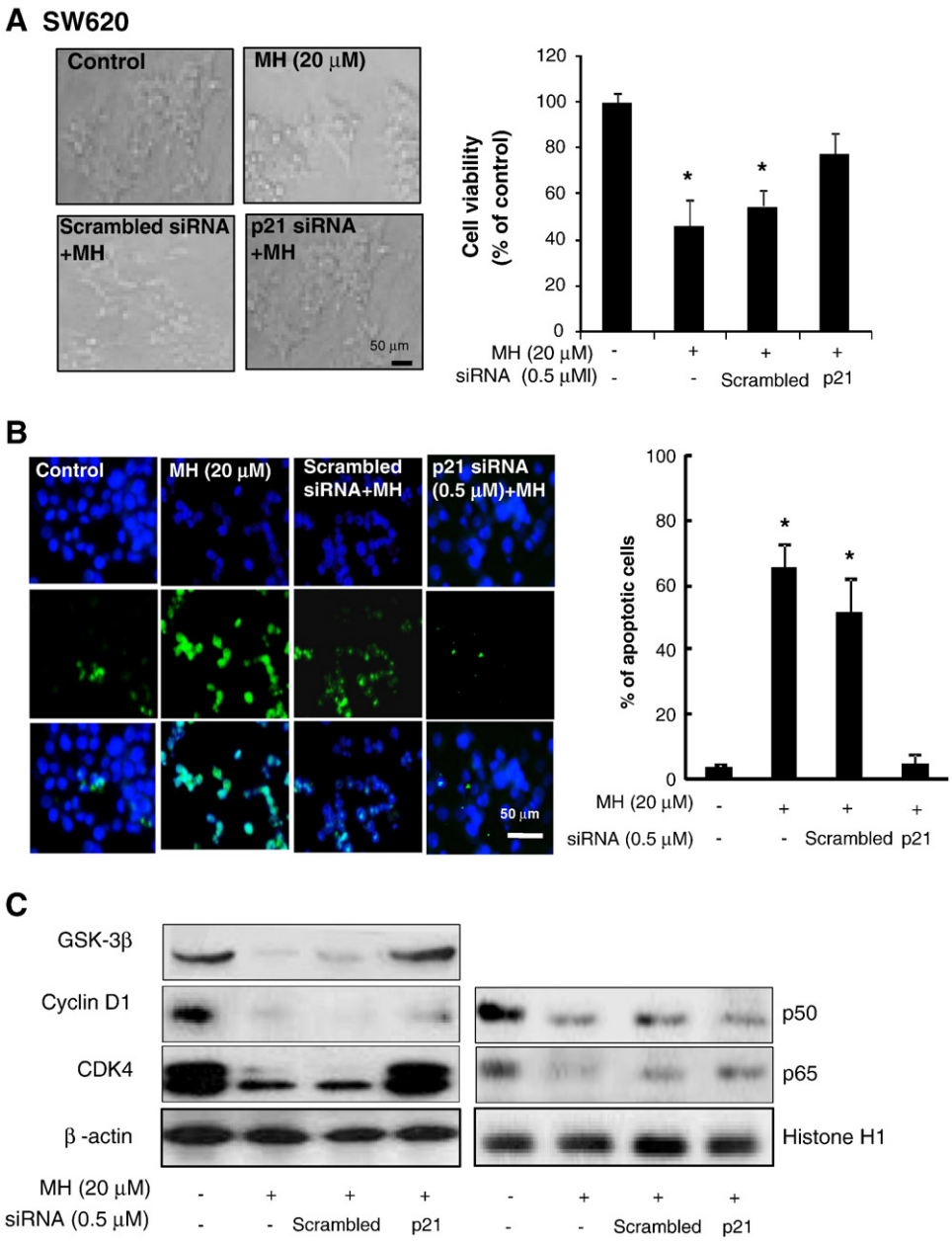

Fig. 4. Abolished effect of transfection of p21 siRNA on the cell viability, apoptosis and related protein expression in MH-treated SW620 colon cancer cells. The cells were transfected with p21 siRNA for 6 h, grown for 24 h in complete media and treated with 20 μM MH for 24 h. (A) Cell counting, (B) DAPI and TUNEL staining and (C) Western blotting were performed as described in Material and methods. *Columns*, mean of triplicate; *bars*, S.D. $^{*}P<.05$ indicates statistically significant differences from the untreated group.

MH-treated tumor tissue (Fig. 6B). Moreover, similar to the inhibitory effect *in vitro*, MH inhibited the NF-κB activity in tumor tissue (Fig. 6C). 4-*O*-methylhonokiol increased the expression of bax and cleaved caspase-3, but decreased the expression of bcl-2 in tumor tissue (Fig. 6D). Immunohistochemical analysis also showed considerable increase in the expression of cleaved caspase-3-positive cells in the MH-treated tumor tissue compared with those of the control group. Apoptotic cell death was also significantly increased in the MH-treated tumor tissue (Fig. 6B). Moreover, p21 expression was also significantly elevated by MH (Fig. 6B and D).

4. Discussion

The present study showed that MH inhibited the growth of colon cancer cells in association with the G_0/G_1 phase cell cycle arrest followed by the induction of apoptotic cell death. 4-*O*-methylhonokiol-induced colon cancer cell growth inhibition is most likely caused by inhibition of the NF-κB activity as well as an increase of p21 expression. Knockdown of NF-κB augmented the effect of MH on cancer cell growth inhibition, apoptotic cell death and expression of cleavage caspase-3 and p21, but knockdown of p21 with siRNA and mutation of p21 abrogated MH-induced NF-κB inactivation, apoptotic cell death as well as expression of CDK4/cyclin D1. These results suggested that MH induced growth inhibition of colon cancer cells via increase of p21expression through the inactivation of NF-κB.

The cancer cell arrested at G_0/G_1 could die by the induction of apoptotic cell death. Nuclear factor-κB is a transcription factor that regulates cell growth and apoptotic cell death [17]. In the present study, MH decreased the NF-κB DNA binding activity and nuclear translocation in colon cancer cells. This correlated well with the

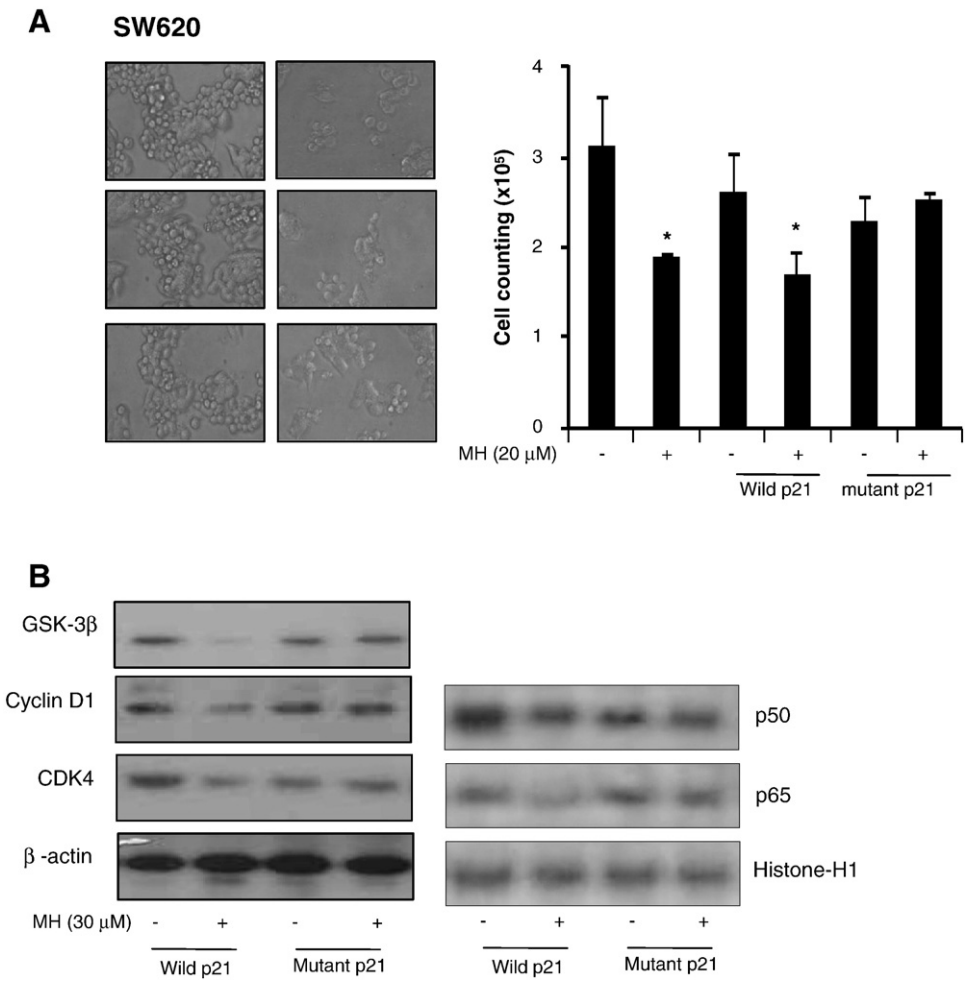

Fig. 5. Abolished effect of transfection of p21 mutant plasmid on the cell viability, apoptotic cell death and related protein expression in MH-treated SW620 colon cancer cells. The cells were treated with p21 mutant plasmid that has mutated cyclin D1/cdk4 complex binding site (Cy1 site, △17–24) for 6 h, grown for 24 h in complete media and treated with 20 μM MH for 24 h. (A) Cell counting and (B) Western blotting were performed as described in Material and methods. *Columns*, mean of triplicate; *bars*, S.D. *$P<.05$ indicates statistically significant differences from the untreated group.

notion that inactivation of NF-κB blocks cancer cell growth via induction of apoptotic cell death. It was found that MH effectively and significantly induced apoptotic cell death in colon cancer cells. Nuclear factor-κB regulates various growth arrest and/or apoptosis regulating target gene expression such as bax, caspase-3, caspase-9, bcl-2, IAP, survivin and GSK-3β [17]. In fact, many naturally occurring anticancer drugs such as curcumin and indole-3-carbinol inhibit cancer cell growth through the regulation of NF-κB target gene expression [34,35]. In agreement with these findings, our data showed that MH clearly inhibited NF-κB-regulated gene products involved in cell proliferation (cyclin D1 and COX-2) and antiapoptotic proteins (c-IAP1/2 and bcl-2), but increased proapoptotic proteins (cleaved caspase-3 and -9, and bax) *in vitro* as well as *in vivo*. Our data are similar to the effect of honokiol and magnolol isolated from *Magnolia officinalis*, which have shown the *in vitro* and *in vivo* antitumor activity of several cancer cells including colon cancer cells through the inhibition of NF-κB activity and NF-κB target gene expression involving carcinogenesis [4,5]. Similarly, we previously found that obovatol, another component isolated from *Magnolia officinalis*, also inhibited colon cancer cells through the inactivation of NF-κB and NF-κB target genes [13]. Moreover, the treatment of MH with the specific inhibitor of NF-κB or knockdown by siRNA of NF-κB augmented MH-induced apoptotic cell death and the expression of cleaved caspase-3 and p21. These data suggest that the inhibitory ability of MH in constitutive activation of NF-κB and its target gene expression is implicated in the MH-induced inhibition of colon cancer cell growth via induction of apoptotic cell death.

The p21 protein regulates G_1/S transition by inhibiting CDKs [31,36]. The present study reveals that MH increased the induction of p21 protein in parallel with the G_0/G_1 cell cycle arrest. However, the MH-mediated apoptotic cell death and G_0/G_1 cell cycle-regulatory proteins cyclin D1 and CDK4 expression are abolished in cells after knockdown of p21 with siRNA. Moreover, transfected cells with cyclin/CDK binding site mutant form of p21 would not respond to MH. 4-*O*-methylhonokiol-induced cell growth inhibition and the inhibition of expression of cyclin D1 and CDK4 as well as cancer cell survival protein GSK-3β were also not observed in p21 mutant cells. This study thus suggests that the induction of p21 expression may be important in the MH-induced arrest G_0/G_1 phase of cell cycle of human colon cancer cells. Similar to our finding, honokiol induces G_0/G_1 cell cycle arrest via induction of p21 in the prostate cancer cells [37]. Nuclear factor-κB is also important in the p21-mediated cell cycle arrest of human breast cancer cells as well as colon cancer cells [27]. Nuclear factor-κB-dependent p21 expression was observed in the resistance of human leukemic cells against chemotherapeutics [28]. In the present study, we found the reciprocal conversable effects of NF-κB and p21 in MH-induced cell growth inhibition and apoptotic cell death. The MH-induced NF-κB

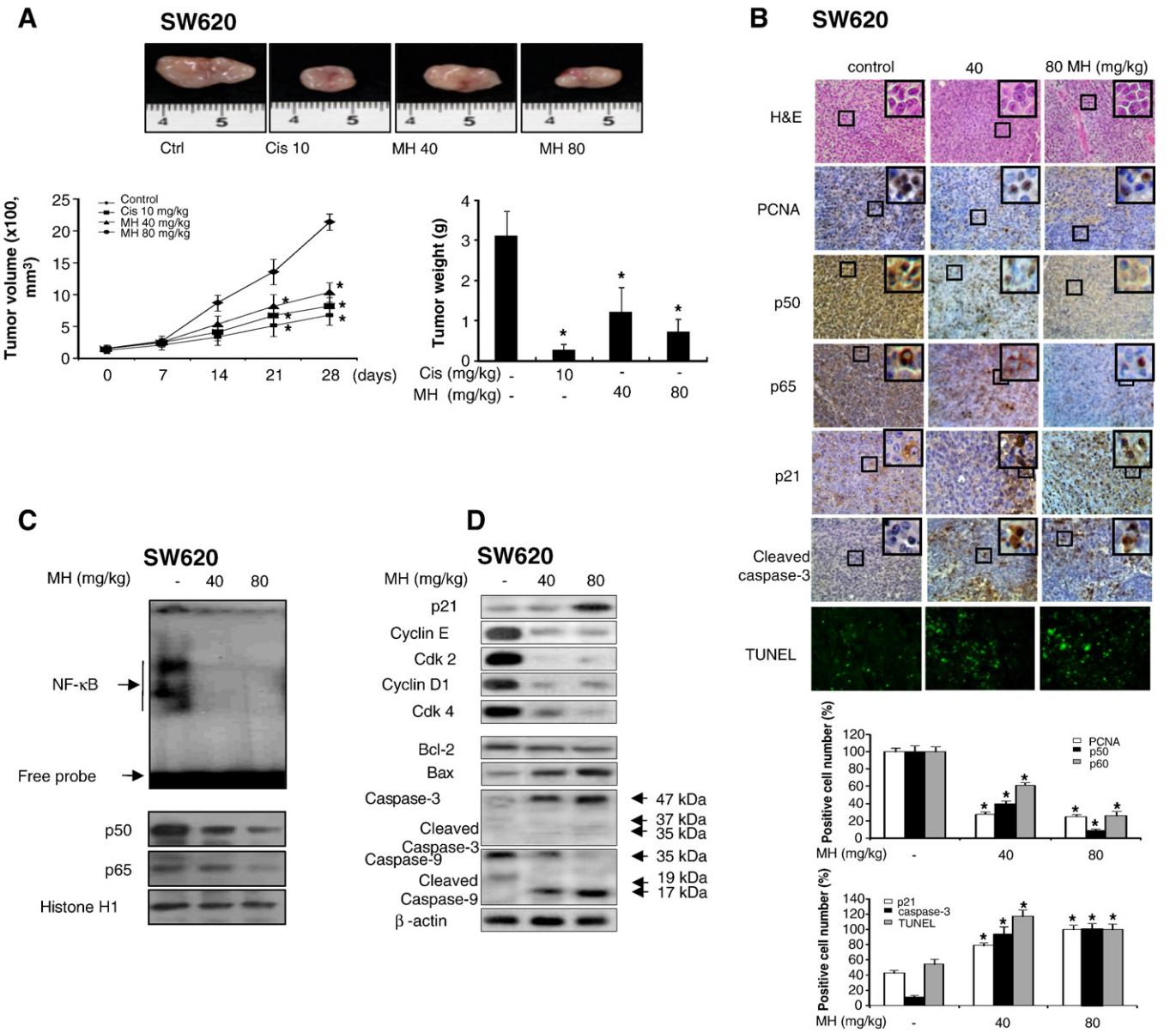

Fig. 6. Effect of MH on the tumor growth in SW620 xenografts *in vivo* model. SW620 and PC3 xenografts mice treated with MH (40 or 80 mg/kg everyday) or cisplatin (10 mg/kg once a week) for 4 weeks. (A) Tumor volume and weight were measured as described in Materials and methods. *Points and columns*, mean of 10 animals; *bars*, S.D. $^{*}P<.05$. (B) Immunohistochemistry, (C) EMSA and (D) Western blotting were performed as described in Materials and methods. *Columns*, mean of three animals; *bars*, S.D. $^{*}P<.05$ indicates statistically significant differences from the untreated group.

inactivation was abolished in cells after knockdown of p21 with siRNA as well as in the cells transfected with mutant p21. Conversely, the treatment of the specific inhibitor of NF-κB or knockdown by siRNA of NF-κB augmented the MH-induced expression of p21 accompanied with apoptotic cell death regulatory proteins (cleaved caspase-3). Moreover, we also found that MH, concomitant with the increase of p21 expression, also increased p21 target genes such as p53 (upstream target) and Rb (downstream target) phosphorylation that could be regulated by NF-κB [38–40]. Supporting our result, a recent study demonstrated that an aqueous extract of *Magnolia officinalis* showed increased p21 expression and decreased NF-κB on human urinary bladder cancer cells [29]. Similar to our findings, several recent studies have shown that anticancer drugs are able to inhibit the growth of several human cancer cells through up-regulation of p21 accompanied by inhibition of NF-κB activation, for example, soy-peptide-treated human breast MCF-7 tumor cells [41], curcumin-treated human breast cancer MDA-MB-231 cells [42] and rhein-lysinate-treated human breast cancer cell SK-Br-3 cells [43]. These data suggest that the inhibitory ability of MH on the constitutive activation of NF-κB could be related with the increased p21 expression, and the modification of cooperative signals is implicated in the MH-induced inhibition of colon and prostate cancer cell growth. Supporting *in vitro* findings, MH increased apoptotic cell death and expression of NF-κB regulatory apoptotic cell death target genes, but inhibited NF-κB activity and the expression of antiapoptotic cell death target genes. 4-*O*-methylhonokiol also increased the expression of p21 with decreased expression of the G_0/G_1 phase of cell cycle regulatory proteins cyclin D1 and E, and CDK2 and CDK4 in both colon cancer xenograft nude mice tumor tissues. These *in vivo* results confirmed the *in vitro* findings demonstrating the significance of p21 and NF-κB signals in the antitumor effect of MH.

The pharmacokinetic data showed that MH can be easily distributed into tissues. We also found that MH has good oral and intestinal absorption as determined by the Caco-2a (data not shown). 4-*O*-methylhonokiol concentrations used in the present *in vivo* study are within the range employed in other previous studies to document antitumor effects of the compounds isolated from *Magnolia officinalis*. Chen et al. reported that ip administration of honokiol at a dose of 80 mg/kg for 31 days displayed significant anticancer activity by 50% inhibition [44]. Treatment with liposomal honokiol at 25 mg/kg reduced the tumor sizes by 42% compared with the untreated tumor in Lewis lung carcinoma-bearing C57BL/6 mice [45]. Thus, the dose used in the present *in vivo* study could be applicable. It was also reported that the *Magnolia officinalis* extract did not show any toxicities in the 21- and 90-day repeated toxicity studies and showed over 240 mg/kg in the no-observed-adverse-effects-level [46], which is about similar to the range of the dose used in the present study if the extract has about 15% of the MH as the our previous study [30]. We also found no weight loss or other toxicities in MH-treated mice. We also evaluated long-term toxicity including carcinogenicity of MH using prediction program (preADME version 1.0.2) and found that it was predicted not to be long-term toxic and carcinogenic to rodents (data not shown). Thus, MH used in the present study could be safe. Collectively, these results suggest that MH should be considered for further clinical investigation to determine its possible chemopreventive and/or therapeutic efficacy against colon cancer in human.

Supplementary materials related to this article can be found online at doi:10.1016/j.jnutbio.2011.03.013.

Acknowledgments

This work was supported by the Korea Research Foundation Grant (MRC, R13-2008-001-00000-00) and by the grant of the Korea Ministry of Education, Science and Technology (The Regional Core Research Program/Chunbuk BIT Research-Oriented University Consortium).

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