

Downregulation of Sp1 is involved in honokiol-induced cell cycle arrest and apoptosis in human malignant pleural mesothelioma cells

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Abstract. Malignant pleural mesothelioma (MPM) is an extremely aggressive type of cancer and is associated with a poor patient prognosis due to its rapid progression. Novel therapeutic agents such as honokiol (HNK) improve the clinical outcomes of cancer therapy, yet the mechanisms involved have not been fully elucidated. The present study examined the regulatory effects of HNK on the growth and apoptosis of MSTO-211H mesothelioma cells and investigated its anticancer mechanism. The results revealed that HNK significantly reduced the cell viability and increased the sub-G₁ population in MSTO-211H cells and suppressed the expression of the specificity protein 1 protein (Sp1). HNK reduced the transcriptional activity of Sp1 regulatory proteins, including cyclin D1, Mcl-1 and survivin, and, thus, induced apoptosis signaling pathways by increasing Bax, reducing Bid and Bcl-xL and activating caspase-3 and PARP in mesothelioma cells. The results suggest that Sp1, a novel molecular target of HNK, may be related to cell cycle arrest and apoptosis induction through the modulation of signal transduction pathways in MPM.

Introduction

Malignant pleural mesothelioma (MPM) is a deadly disease that arises from the mesothelial layer of the pleural cavity and afflicts 2,000 to 3,000 individuals every year in the US (1,2). MPM has been linked to asbestos, iron and Simian

virus 40 exposure (3-5) and represents a worldwide concern since its incidence has greatly increased during the last three decades (6). MPM progresses rapidly and is extraordinarily debilitating, particularly in the later stages. In addition, its poor prognosis stems from an overall survival period of only 9-17 months from the initial diagnosis (2). An aggressive multimodality therapy for MPM can offer survival benefits not only for a small subset of patients in the early stages of the disease but also for terminal patients with unresectable tumors who are not responsive to current therapeutic approaches (2,7). This suggests an urgent need for new therapeutic targets and effective therapies for improving the prognosis of MPM. Several studies have reported that novel plant-derived compounds act as antitumor agents through the modulation of biological pathways (8). This suggests that chemotherapeutic agents used in the treatment of MPM may activate apoptosis pathways.

The present study focused on honokiol (HNK) as this anti-cancer agent is widely used as the drug of choice in treating cancer. HNK, a biologically active biphenolic compound isolated from the *Magnolia* species, has multiple medicinal uses against oxidative stress, microbial infection and anxiety, among others (9). Noteworthy, HNK demonstrates antineoplastic properties *in vitro* against various types of cancers and is effective *in vivo* in treating angiosarcoma (10), colorectal carcinoma (11), breast cancer (12) and gastric cancer (13). Several studies have suggested that the cytotoxic effect of HNK is caused by the predominant activation of distinct apoptotic mechanisms in tumor cells (10,11,14-16). Apart from apoptosis, the suppression of cancer cell proliferation is one of the most important strategies for antitumorogenesis.

Specificity protein 1 (Sp1), a constitutive transcription factor, plays an important role in various physiological processes such as cell cycle regulation, apoptosis and angiogenesis (17,18). In this regard, many studies have documented that Sp1 activity and expression levels are increased in various types of human cancers including prostate cancer, pancreatic adenocarcinoma, thyroid cancer, hepatocellular carcinomas, lung cancer, colorectal cancer, gastric cancer and breast cancer, but not in normal tissues (19,20).

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Therefore, the present study examined the regulatory effects of HNK on the growth and apoptosis of MSTO-211H mesothelioma cells and investigated its anticancer mechanism in relation to Sp1.

Materials and methods

Cell culture and transfection. MSTO-211H cells obtained from the American Tissue Culture Collection (Manassas, VA) were maintained at 37°C in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin in the presence of 5% CO₂. For transient transfection, 1.4x10⁶ cells were plated onto a 60-mm cell culture dish, grown overnight, and transfected with DNA using Lipofectamine (Invitrogen).

MTS assay. The effects of HNK on cell viability were determined using the CellTiter 96 Aqueous One Solution cell proliferation assay kit (Promega, Madison, WI) according to the manufacturer's instructions. MSTO-211H cells were seeded onto a 96-well plate for 24 h and then treated with HNK for 24 and 48 h. The MTS solution was analyzed using the GloMax-Multi Microplate Multimode reader (Promega) at 482 and 690 nm.

DAPI staining. The number of cells undergoing apoptosis following treatment with HNK was quantified using DAPI staining. Cells with nuclear condensation and fragmentation were determined using the nucleic acid stain 4'-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Following a 48-h treatment of HNK at different concentrations (2, 4 and 6 µg/ml), MSTO-211H cells were harvested and fixed in 100% methanol at room temperature for 20 min. The cells were seeded on slides, stained with DAPI (2 mg/ml), and then monitored using a FluoView confocal laser microscope (FluoView FV10i, Olympus Corp., Tokyo, Japan).

Propidium iodide staining. Following HNK treatment of the MSTO-211H cells, detached cells were collected separately, and adherent cells were dissociated by trypsin-EDTA. The cells were washed with cold PBS and then pooled and centrifuged before being fixed in 70% ethanol overnight at -20°C. Prior to the flow cytometric analysis, the cells were centrifuged and incubated for 30 min at 37°C in PBS to allow for the release of low-molecular-weight DNA. After centrifugation, cell pellets were resuspended and treated with 150 mg/ml RNase A and 20 mg/ml propidium iodide (PI) using a MACSQuant Analyzer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Western blot analysis. MSTO-211H cells treated with HNK were washed three times with ice-cold phosphate-buffered saline (PBS) and harvested in an ice-cold PRO-PREP™ protein extraction solution (Intron Biotechnology, Inc., Korea) containing a protease inhibitor. Protein concentrations were measured using the Bradford protein assay. Protein samples were separated on an SDS-PAGE gel and transferred to a PVDF membrane (Immobilon-P, Millipore) using a semi-dry blotting apparatus. ECL western blotting was performed according to the manufacturer's instructions (Thermo Scientific, Rockford, IL).

In vitro pull-down assays. Cell lysates were incubated with HNK-Sepharose 4B (or Sepharose 4B only as a control) beads (50 µl, 50% slurry) in reaction buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, 2 µg/ml bovine serum albumin, 0.02 mM phenylmethylsulfonyl fluoride and 1X protease inhibitor cocktail). After incubation with gentle rocking overnight at 4°C, the beads were washed five times with a buffer solution (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40 and 0.02 mM phenylmethylsulfonyl fluoride) and proteins bound to the beads were analyzed by western blotting using the anti-Sp1 antibody.

Luciferase assay. MSTO-211H cells were grown to 50-80% confluence on a 60-mm cell dish and were transfected with Lipofectamine (Invitrogen) with 1 µg each of cyclin D1-luc, Mcl-1-luc and survivin-luc and 0.5 µg of pCMV/β-gal plasmids. The luciferase reporter assay kit from Promega was used following the manufacturer's protocol. Luciferase activity was measured with the Lumat LB 9507 luminometer (EG&G Berthold). The values shown represent an average of three experiments for each sample.

Statistical analysis. The statistical significance of differences was assessed using the Student's t-test. A P-value <0.05 relative to the control was considered to indicate a statistically significant result.

Results

Growth inhibitory effect of HNK on a human mesothelioma cell line. We investigated the effects of HNK on a human mesothelioma cell line. The structure of HNK is shown in Fig. 1A. To ascertain the efficiency of HNK as an anticancer drug, the effects of HNK were tested using an MTS assay using MSTO-211H cells. As shown in Fig. 1B, the efficiency of HNK in altering the cell viability of MSTO-211H cells was assayed after 24 and 48 h of incubation in HNK-containing medium at different concentrations (2, 4 or 6 µg/ml). The viability curves revealed that HNK reduced MSTO-211H cell viability at 24 and 48 h, in a concentration-dependent manner (P<0.05). In particular, a maximum reduction in cell viability was observed at 48 h. Apoptosis-induced changes in the cell morphology in the HNK-containing medium were observed. After 48 h, the apoptotic phenotype showed cell rounding, cytoplasmic blebbing and irregularities in shape, indicating a sharp increase in the apoptosis of HNK-treated MSTO-211H cells in a concentration-dependent manner (Fig. 1C).

HNK induces cell cycle arrest at the G1 phase and apoptosis in a human mesothelioma cell line. Cancer cell growth can be suppressed by cell cycle arrest or apoptosis induction or both (8). Morphological changes induced by HNK were examined following treatment of MSTO-211H cells with HNK at a concentration of 2, 4 and 6 µg/ml for 48 h. In the DAPI staining analysis, MSTO-211H cells were observed via fluorescence microscopy following exposure to DAPI, which specifically stains nuclei. The results indicated the presence of nuclear condensation and perinuclear apoptotic bodies in the MSTO-211H cells following HNK treatment at concentra-

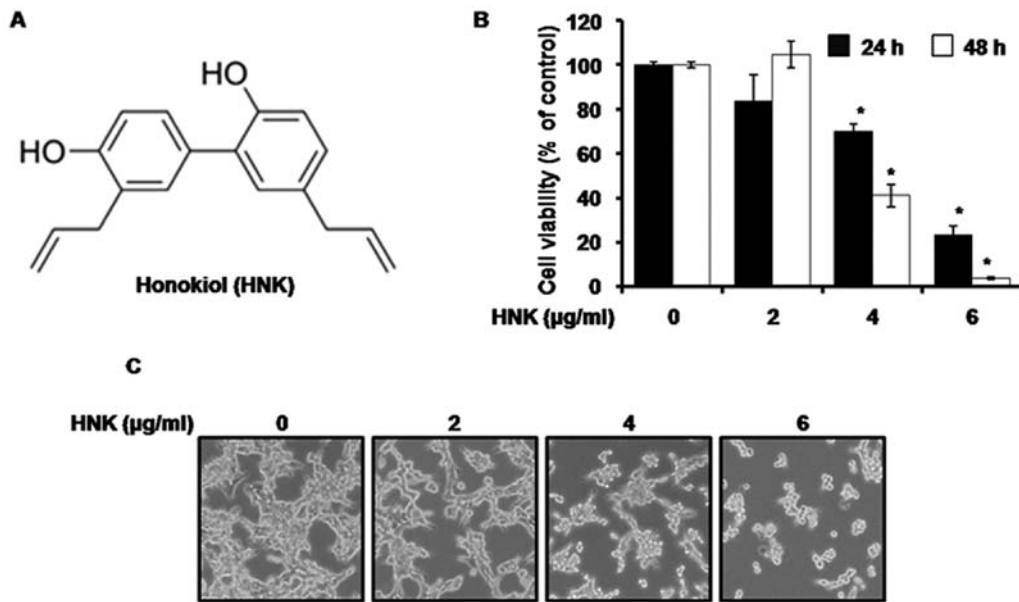


Figure 1. HNK treatment induces the apoptosis of MSTO-211H cells. (A) Chemical structure of HNK. (B) The cell viability of HNK-treated MSTO-211H cells was assessed using an MTS assay as described in Materials and methods. Data represent the mean percentage levels \pm SD. * $P < 0.05$, significantly different compared with the DMSO-treated control by paired t-test ($n=3$). (C) Morphological changes in MSTO-211H cells at 48 h following treatment with HNK at concentrations of 2, 4 and 6 $\mu\text{g/ml}$.

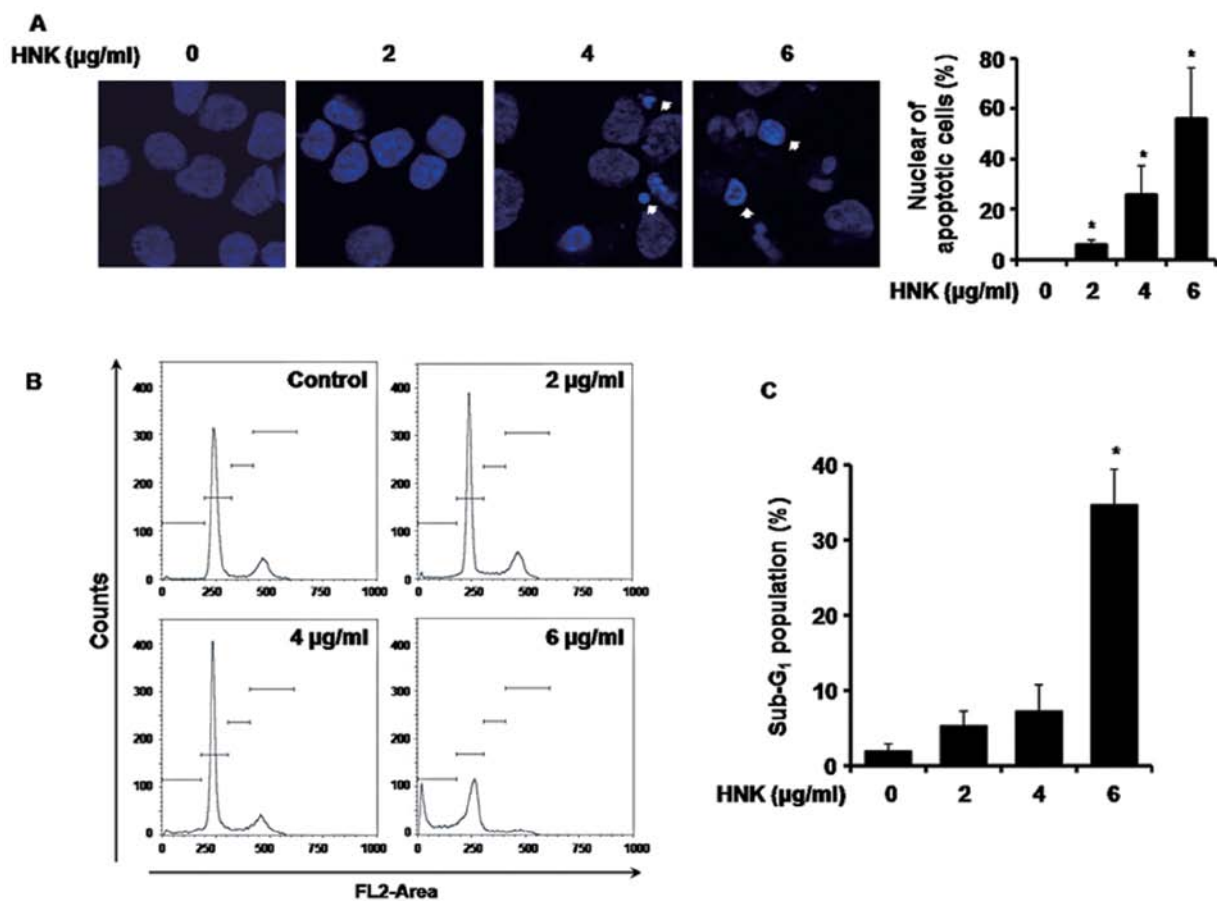


Figure 2. Effects of HNK on the proliferation of MSTO-211H cells. (A, left) MSTO-211H cells were cultured without (control) or with HNK (2, 4 and 6 $\mu\text{g/ml}$) for 48 h in each case. Images of DAPI-stained MSTO-211H cells are based on fluorescence microscopy (magnification, $\times 600$). White arrows indicate condensed nuclei. (A, right) DNA fragmentation and nuclear condensation were quantified, and the data represent the mean percentage levels \pm SD ($n=3$; * $P < 0.05$). (B) MSTO-211H cell cultures were treated with 2, 4 and 6 $\mu\text{g/ml}$ HNK or PBS (vehicle). The cells were then washed, fixed and stained with PI and analyzed for DNA content through FACS analysis 48 h after treatment. Ratios of apoptotic cells were measured by FACS analysis after PI staining. (C) The data represent the percentage of the G_0 - G_1 population in MSTO-211H cells following a 48-h treatment with DMSO (control) or 2, 4, and 6 $\mu\text{g/ml}$ HNK. * $P < 0.05$, significantly different compared with DMSO-treated control by paired t-test. Repeated experiments produced similar results ($n=3$).

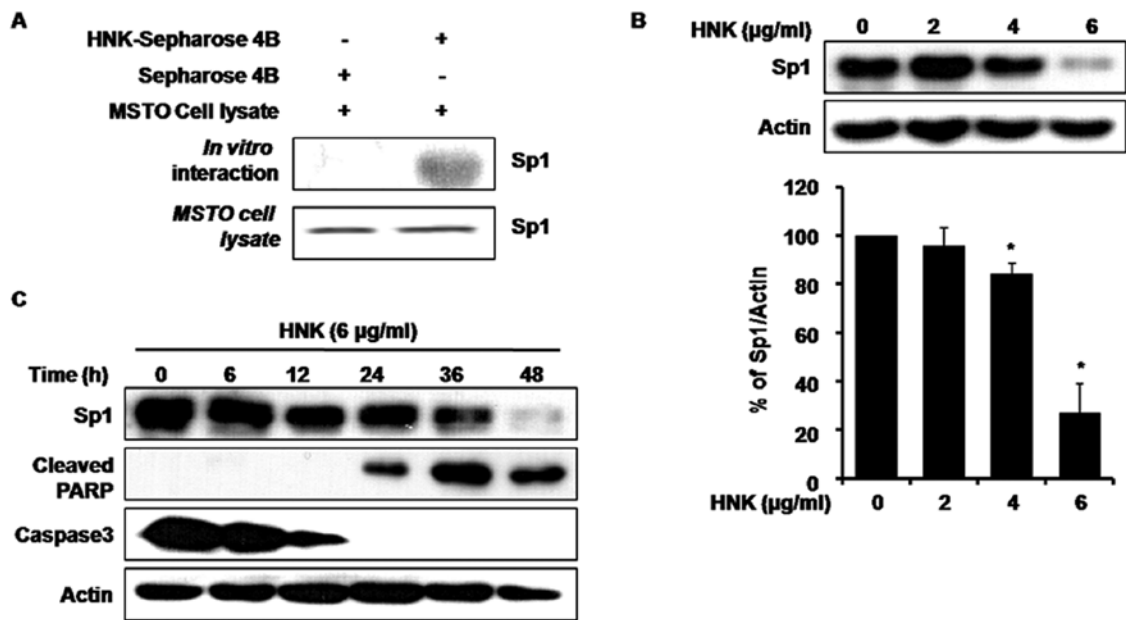


Figure 3. HNK binds to Sp1 and suppresses Sp1 levels via apoptosis. (A) MSTO-211H cell lysates were prepared, subjected to a pull-down assay using HNK-Sepharose 4B beads (or only Sepharose 4B as a control), and subjected to western blot analysis. (B) MSTO-211H cells were treated with 2, 4 and 6 $\mu\text{g/ml}$ HNK for 48 h, and whole-cell extracts were prepared and separated on SDS-PAGE and then subjected to western blotting with an anti-Sp1 antibody. Actin was used as a loading control. The graphs indicate the ratio of Sp1 to actin expression. (C) Experiments to assess the time-dependent effects of HNK on Sp1, cleaved PARP, and caspase-3 expression were performed using MSTO-211H cells treated with 6 $\mu\text{g/ml}$ HNK for 0, 6, 12, 24, 36 and 48 h.

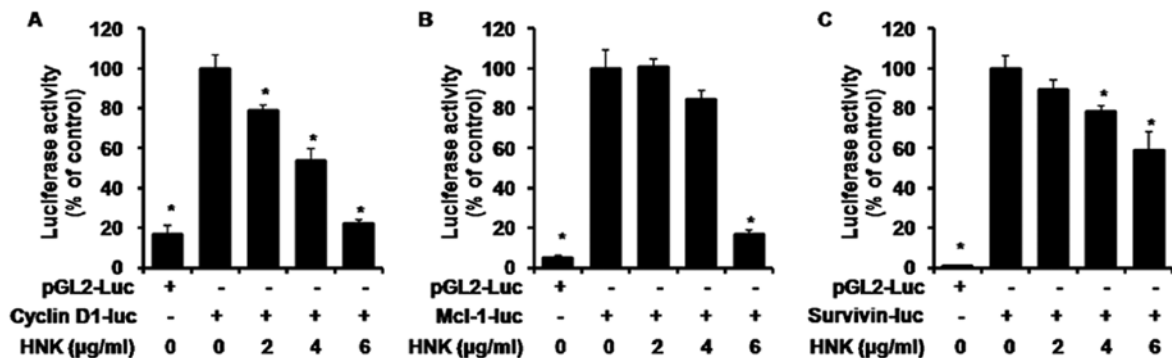


Figure 4. Effects of HNK on downstream target proteins regulated by Sp1. MSTO-211H cells were transfected with the (A) cyclin D1-luciferase (cyclin D1-luc), (B) Mcl-1 luciferase (Mcl-1-luc), and (C) survivin-luciferase (survivin-luc) plasmids, incubated for 24 h, and treated with 2, 4, and 6 $\mu\text{g/ml}$ HNK for 48 h. Then whole-cell extracts were prepared and analyzed for luciferase activity. The results shown are representative of three independent experiments ($P < 0.05$).

tions of 4 and 6 $\mu\text{g/ml}$ for 48 h (Fig. 2A). The percentage of apoptosis was determined to be 6 ± 1.6 , 26 ± 11.3 and $20 \pm 20.3\%$ at concentrations of 2, 4 and 6 $\mu\text{g/ml}$ HNK compared with the untreated control cells.

For the determination of whether the HNK-mediated growth inhibition of MSTO-211H cells was attributable to cell cycle arrest, cell cycle distribution was analyzed by FACS analysis. As shown in Fig. 2B, a significant increase in the number of sub- G_1 cells was noted when compared with the control (5.3 ± 1.9 , 7.2 ± 3.5 and $34.7 \pm 4.6\%$ in the presence of 2, 4 and 6 $\mu\text{g/ml}$ HNK, respectively, in MSTO-211H cells). The histogram in Fig. 2C shows the quantification of FACS data.

HNK suppresses Sp1 expression in a human mesothelioma cell line. The potential interaction between HNK and apoptosis-related proteins was examined since HNK has been

found to have marked effects on apoptosis and cell cycle arrest in various cancer cell lines. Therefore, an *in vitro* pull-down assay was performed using HNK-conjugated Sepharose 4B beads. Sp1 was found to bind to HNK-conjugated Sepharose beads but not to Sepharose beads alone (Fig. 3A), providing support for the direct interaction between HNK and Sp1 and suggesting that Sp1 may be a possible target of HNK in human MSTO-211H cells. The effects of HNK treatment on the levels of Sp1 were examined by western blotting. As shown in Fig. 3B, HNK treatment led to a sharp decrease in the level of Sp1 in MSTO-211H cells at 48 h after treatment.

In order to better characterize the apoptotic action of HNK, the cleavage of the DNA repair enzyme poly(adenosine diphosphate-ribose) polymerase (PARP) as a substrate for activated caspase-3 and caspase-3 expression levels were confirmed by western blotting (Fig. 3C). There were increases

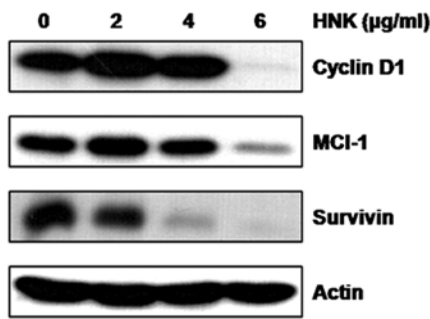


Figure 5. HNK suppresses Sp1 regulatory proteins involved in proliferation and survival. MSTO-211H cells were treated with 2, 4 and 6 µg/ml HNK for 48 h, and whole-cell extracts were prepared and then resolved on SDS-PAGE. The membrane was sliced according to the molecular weight and probed using anti-cyclin D1, anti-Mcl-1 and anti-survivin antibodies. Actin was employed as a loading control. The results shown are representative of three independent experiments.

in cleaved PARP and activated caspase-3 following HNK (6 µg/ml) treatment of MSTO-211H cells in a time-dependent manner.

HNK modulates regulators of cell cycle arrest and apoptosis. Sp1 activation has been shown to regulate the expression of various gene products involved in cell survival and proliferation (18,21). Based on the results for DNA fragmentation and cell cycle distribution, the potential relationship between the induction of apoptosis and cell cycle arrest by HNK and the transcriptional activity of cell cycle regulatory proteins and apoptosis-related transcription factors was examined. We investigated the effects of HNK on cell cycle regulator cyclin D1, anti-apoptotic proteins Mcl-1 and survivin dependent gene transcriptions. When the MSTO-211H cells transiently transfected with cyclin D1-luc (Fig. 4A), Mcl-1-luc (Fig. 4B), and survivin-luc (Fig. 4C) constructs were treated with 2, 4 and 6 µg/ml HNK, significant decreases in transcriptional activity mediated by these proteins were observed in a dose-dependent manner. The cyclin D1, Mcl-1 and survivin protein expression levels were examined using western blotting. The results indicated that these levels decreased gradually after HNK treatment (Fig. 5).

HNK alters the expression of proteins associated with the cell cycle and survival. The expression of genes involved in the regulation of apoptosis was analyzed. The apoptotic cell death by HNK was well correlated with the activation of caspase-3 through the cleavage of the pro-form during HNK treatment. The upregulation and downregulation of Bax and Bid, respectively, and Bcl-xL expression appeared to be involved in apoptotic cell death. In addition, PARP cleavage was induced by HNK (Fig. 6). These results indicate that downregulation of cyclin D1, Mcl-1 and survivin and activation of caspase-3 were involved in the HNK-mediated growth arrest and apoptotic cell death in MSTO-211H cells.

Discussion

Compounds of natural origin such as topotecan, irinotecan, etoposide, teniposide and paclitaxil are widely employed in

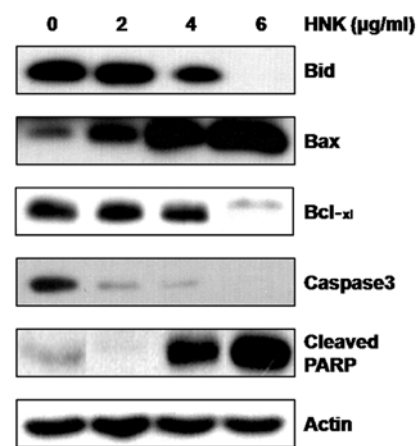


Figure 6. The effects of HNK on the apoptosis of MSTO-211H cells. MSTO-211H cells were treated with 2, 4 and 6 µg/ml HNK for 48 h, and whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to western blotting using anti-Bid, anti-Bax, anti-Bcl-xL, anti-caspase-3 and anti-cleaved PARP antibodies. Actin was employed as a loading control. The results shown are representative of three independent experiments.

clinical trials as chemotherapeutic agents (22). Therefore, the evaluation of natural compounds with anticancer properties has been considered to play an important role in the development of anticancer drug. The present study focused on the anticancer effects of HNK, a pharmacologically active component found in the traditional Chinese medicinal herb, *Magnolia* species. Previous studies have demonstrated the anticancer properties of HNK, including cell cycle arrest, apoptosis and necrosis in a variety of cancer cell lines such as human breast cancer (23), human colorectal carcinoma RKO (24), human squamous lung cancer CH27 (15), human pancreatic cancer (25), adult T-cell leukemia (26) and hepatocellular carcinoma cells (27). This suggests that HNK may be involved in the apoptotic pathway. However, the exact mechanism underlying the anticancer activity of HNK remains unclear.

In this study, our findings along with the findings of previous research, suggest that HNK may induce apoptosis in human MPM cells. In this study, the apoptotic effects of HNK on MSTO-211H cells were examined through an MTS assay. This assay, a traditional method for discovering anti-cancer drugs, can be used to determine the cytotoxic effect and proliferation of cell lines (28,29). In this study, this assay was used to elucidate the apoptotic effects of HNK on MSTO-211H cells (Fig. 1).

As shown in Fig. 2, HNK inhibited the proliferation of MSTO-211H cells through cell cycle arrest at G₀/G₁ and induction of apoptosis.

Sp1, a transcription factor, is overexpressed in various human cancer cell lines (30-34) and plays a role in the regulation of genes involved in most cellular processes (35). In this regard, Sp1 may exhibit transcriptional activity for promoters of genes involved in the progression, differentiation and oncogenesis of the cell cycle (19). In addition, Sp1 may be inhibited by compounds of natural origin with anticancer properties. For example, recent studies have demonstrated that flavonoids suppress Sp1 expression (36) and, thus, inhibit the known Sp1 target genes cyclin D1, Mcl-1 and survivin in a selective

manner (37-41). The results of the present study demonstrated the potential chemotherapeutic properties of HNK. That is, HNK suppressed Sp1 expression via direct interactions with Sp1, preventing Sp1 from binding to G-C-rich promoters. In addition, HNK inhibited the transcriptional activity and expression of Sp1 downstream proteins, including cyclin D1, Mcl-1 and survivin, in a dose-dependent manner (Figs. 4 and 5). HNK reduced Bid and Bcl-xL, increased Bax, and activated caspase-3 and PARP, suggesting that HNK regulated Sp1 and ultimately led to apoptotic cell death (Fig. 6).

The results regarding the chemopreventive effect of HNK as a compound of natural origin on MPM cells revealed that HNK downregulated Sp1 expression and Sp1 target transcription factors, including cyclin D1, Mcl-1 and survivin, thereby inducing the apoptosis of MSTO-211H cells. To conclude, this study is the first to suggest that the apoptosis induced by HNK in MSTO-211H cells may be mediated by the downregulation of Sp1. The results suggest that the antiproliferative and apoptotic effects of HNK are mediated through the suppression of Sp1-regulated gene products.

Acknowledgements

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