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Dealcoholized Korean Rice Wine *(Makgeolli)* Exerts Potent Anti-Tumor Effect in AGS Human Gastric Adenocarcinoma Cells and Tumor Xenograft Mice

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Makgeolli is a traditional wine in Korea and has been traditionally believed to exhibit health benefits. However, the inhibitory effect of dealcoholized *makgeolli* (MK) on cancer has never been investigated scientifically. In this study, MK exhibited an anti-angiogenic effect by inhibiting tube formation in human umbilical vein endothelial cells, without cytotoxicity. Treatment with MK reduced the proliferation of AGS human gastric adenocarcinoma cells in a dose-dependent manner and increased the sub-G1 population. Next, we evaluated whether MK could induce apoptosis in AGS cells by using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay or Annexin V method. Treatment with MK at 500 and 1,000 μg/ml increased the number of TUNEL-positive AGS cells. Under the same conditions, MK-treated (500 and 1,000 μg/ml) cells showed significant induction of early or late apoptosis, compared with untreated cells (no induction). In addition, MK also induced phosphatase and tensin homolog (PTEN) expression in AGS cells. However, p53 expression in AGS cells was not changed by MK treatment. Furthermore, MK at 500 mg/kg·d reduced the tumor size and volume in AGS tumor xenografts. Taken together, MK may be useful for the prevention of cancer cell growth.

Keywords: *Makgeolli*, anti-cancer, anti-angiogenesis, AGS human gastric adenocarcinoma cells, tumor xenograft

Introduction

Gastric cancer is a major obstacle to the increasing life expectancy worldwide and poses a serious risk of death. Generally, the primary therapy for gastric cancer is to surgically remove the cancer and perform chemotherapy after surgery. However, the major problem of eventual death of the patients remains to be resolved. For this reason, the prevention of gastric cancer is more important than surgery after cancer development. Previous studies have revealed that natural products and their active ingredients have anti-cancer effects and could serve as a novel strategy for repression of cancer development [10, 15]. For example, the results of previous studies suggest that flavonoids derived from plants are beneficial in the prevention of tumor progression *via* the modulation of anti- or pro-apoptotic proteins [10, 15, 26]. Previous study also investigated the anti-cancer effect of ginsenosides derived from *Panax ginseng* in human cancer cells, with a focus on the role of anti- or pro-apoptotic proteins [26]. Furthermore, a number of studies have demonstrated that tumor suppressor proteins, such as phosphatase and tensin homolog (PTEN) and p53, are important targets of natural products or their active ingredients for cancer prevention [5, 9, 15, 23, 24].

PTEN is a well-known tumor suppressor protein that consists of two domains; the phosphatase domain and the C2 domain [16, 17]. PTEN plays a central role in the PI3 kinase pathway by dephosphorylating the 3′-phosphate of the inositol ring in phosphatidylinositol (3,4,5)-trisphosphate

(PtdIns(3,4,5)P_3 or PIP_3). In addition, PTEN functions as a preventative target for cancer development by inhibiting uncontrolled cell growth, formation of tumors, and tumor metastasis [16, 17]. Once mutated, PTEN becomes inactive, thereby resulting in increased cell proliferation and reduced cell death. This can lead to the development of tumors in a variety of cancers [16]. Thus, the induction of PTEN expression by therapeutic agents or natural ingredients is also a strategy for preventing cancers [1, 6, 21, 23]. Several papers have supported the importance of PTEN for cancer prevention by treatment with natural products, including neem leaf and silymarin, which induce expression of pro-apoptotic genes, including PTEN [1, 21]. Interestingly, red wine constituents, including resveratrol, have been reported by many investigators to exhibit inhibitory effects on cancer growth *via* PTEN expression [2, 23].

Makgeolli is a traditional rice wine brewed in Korea with *nuruk*, rice, and yeast. *Nuruk,* made from wheat or grits, plays a role in the degradation of rice starch during fermentation, and is used as a growth source for microorganisms such as fungi, yeast, and a variety of other bacteria [9]. Recently, a number of papers have proposed that *makgeolli* contains various ingredients such as proteins, sugars, vitamins, bioactive compounds, and organic acids, which are believed to play a central role in health benefits [8, 9]. However, the inhibitory effects of dealcoholized *makgeolli* on gastric cancer are yet to be elucidated. For this reason, we investigated whether dealcoholized *makgeolli* induces apoptotic cell death in AGS cells. We found that the induction of apoptosis by dealcoholized *makgeolli* is accompanied by PTEN, but not p53, expression.

Materials and Methods

Cell Lines and Materials

AGS human gastric adenocarcinoma cells and human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from WelGene (Daegu, South Korea). The cells were cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin and were maintained in a 5% CO_2 incubator at 37°C. Antibodies against PTEN and p53 were purchased from Millipore, and beta-actin was purchased from Bethyl Laboratories, Inc. (Montgomery, TX, USA). Horseradish peroxidase-conjugated secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). (4,5-Dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA). The angiogenesis assay kit, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit, and Annexin V/Dead Cell kit were purchased from Millipore (Darmstadt, Germany). The WST-1 kit was purchased from Enzo Life Sciences (Farmingdale, NY, USA).

Makgeolli Brewing and Sample Preparation

Makgeolli brewing was performed to two steps; in the first step, *nuruk* (3 kg), sterilized water (20 L), steamed rice (10 kg), and dry yeast (5 g) were mixed and fermented at 25°C for 2 days. To prepare mash, washed non-glutinous rice (20 kg) was soaked for 3 h, drained for 30 min, and steamed for 45 min. After cooling, the steamed rice and 40 L of water were added to the product of the first step, and were then mixed and fermented at 25°C for 7 days in the second step. After 7 days, the fermentation broth was strained through a sieve to produce *makgeolli*. To prepare dealcoholized *makgeolli* (MK), alcohol was removed from the *makgeolli* by evaporation in a rotary evaporator (Laborota 4000; Heidolph Instruments Inc., Schwabach, Germany) and freeze-drying. The resulting MK powder was resuspended in distilled water prior to the cell culture and animal experiments.

Angiogenesis Assay

Tube formation by endothelial cells was measured using an *in vitro* angiogenesis assay kit according to the manufacturer's instructions. Ninety-six-well culture plates were coated with 50 µl of Matrigel, and then HUVECs were plated at a density of 9×10^4 cells/well on the upper phase of the Matrigel. For evaluating inhibitory activity on tube formation, the MK was premixed with a cell suspension before adding the cells on top of the Matrigel. Matrigel cultures were incubated at 37°C for 4 h. Tube formation was observed using a phase-contrast microscope (Olympus, Tokyo, Japan) and digitally imaged.

Determination of Cell Viability

HUVECs were treated with the same conditions as mentioned above. Cells were mixed with 10 µl of WST-1 reagent for 4 h at 37°C. After incubation, absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) reader. AGS cells were seeded in 24-well plates with MK for 72 h and then incubated with 10 µl of MTT solution (5 mg/ml in phosphate-buffered saline (PBS)) for 2 h at 37°C. The cells were exposed directly to DMSO after discarding the medium, and 100 µl aliquots of purple supernatants were transferred from the 24-well plate to a 96-well plate. Relative cell viability was determined using an absorbance reader.

TUNEL Assay

Cells were treated with vehicle or 100, 250, 500, and 1,000 µg/ml MK for 48 h. The TUNEL assay kit was used according to the manufacturer's instructions. Cells positive for TUNEL, which

indicates DNA fragmentation, were detected by fluorescence microscopy (Nikon, Tokyo, Japan).

Apoptosis

The percentages of apoptotic cells were detected using the Annexin V/Dead Cell kit and Muse analyzer (Millipore). Cells were seeded and treated with MK (100, 250, 500, and 1,000 μg/ml) for 48 h. Thereafter, treatment media were collected using trypsin-EDTA and washed with PBS. The harvested cells were centrifuged at 12,000 rpm for 2 min and resuspended with complete medium. Each resuspended cell was mixed with 100 μl of MUSE Annexin V cell reagent. After incubation for 20 min in the dark, the stained cells were determined according to the manufacturer's protocol.

Cell Cycle Assay

MK-induced apoptotic AGS cells were collected and fixed with 70% ethanol in PBS. Fixed cells were centrifuged and washed again with PBS. Then, the cells were stained with PI and Rnase A. Sub-G1 populations were sorted by fluorescence-activated cell sorting (FACS) at 488 nm and measured using ModFit LT ver. 3.3.11.

Protein Extraction and Western Blot Analysis

Cells were washed with PBS and lysed with lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM phenylmethanesulfonylfluoride, protease inhibitor cocktail, and phosphatase inhibitor cocktail). Twenty micrograms of proteins was separated with SDS-polyacrylamide gel electrophoresis. After transferring the proteins to the nitrocellulose paper, blocking was performed with 5% BSA at room temperature. The membrane was incubated overnight with the primary antibodies, washed with Tris-buffered saline with Tween 20, and incubated with a secondary antibody for 1 h. Protein expression was detected using a chemiluminescence system.

Tumor Xenograft Model

Five-week-old male nude mice were purchased from Raon Bio (Yongin, Republic of Korea). The mice were randomly divided into three groups: None (untreated); induction group (AGS cell inoculation); MK (AGS cell inoculation with MK at 500 mg/kg·d). AGS cells (2×10^6 cells/100 μl) were mixed with 100 μl of Matrigel and the mixture was subcutaneously injected into both sides of the flanks of the four mice per group respectively. After one week, the mice were intraperitoneally injected with MK at a dosage of 500 mg/kg for 7 weeks. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Korea Food Research Institute.

Statistical Analysis

All data were expressed as the mean ± SD. Data were analyzed with nonparametric methods using the SPSS computer-based statistics program (ver. 20). Analyses for statistical differences were performed using Student's *t*-test. The level of statistical significance was considered as $p < 0.05$.

Results

Effect of Dealcoholized *Makgeolli* on HUVEC Tube Formation

Because angiogenesis is correlated with cancer development [18], we first investigated the effects of MK on tube formation related to angiogenesis of HUVECs, by using a tube formation assay. This assay is a well-known tool for *in vitro* angiogenesis evaluation. As shown in Fig. 1A and 1B, treatment with MK in the concentration range of 500–1,000 μg/ml significantly inhibited tube formation in HUVECs as compared with that in the vehicle controls (0.5% DMSO). In addition, we also examined the cytotoxicity of MK on HUVECs by using the WST-1 assay. As shown in Fig. 1C, there were no significant differences in cytotoxicity between the control and MK-treated groups. These results demonstrate that MK can reduce angiogenesis without cytotoxicity.

MK Attenuates Cancer Cell Proliferation and Increases Sub-G1 Populations in AGS Cells

We next examined the effects of MK on the proliferation of AGS cancer cells. Treatment with MK significantly reduced cancer cell proliferation in a dose-dependent manner compared with that of the vehicle controls (Fig. 2A). Under the same conditions, MK also increased the sub-G1 population in a dose-dependent manner compared with that of the vehicle controls (Fig. 2B). Therefore, MK is effective for the inhibition of cancer cell proliferation under cell culture systems.

MK Increases TUNEL- and Annexin V-Positive AGS Cells

MK had the potential to inhibit cancer cell proliferation; therefore, we examined whether MK-induced cell death was accompanied by an apoptotic motif in AGS cells. We performed the TUNEL assay and Annexin V staining. MK increased TUNEL-positive cells in a dose-dependent manner (Fig. 3A). In addition, it increased Annexin V-positive cells compared with the total apoptotic cells (Fig. 3B). Collectively, our data demonstrate that MK is effective for inhibition of tumor cell growth and induction of apoptosis in AGS cells.

PTEN Expression Is Involved in the Treatment of MK

It is well established that tumor suppressor proteins such as PTEN and p53 are important for suppression of cancer [14, 16]; therefore, induction of tumor suppressor proteins has been suggested as a strategy for cancer prevention. Thus, we next investigated whether PTEN and p53 are

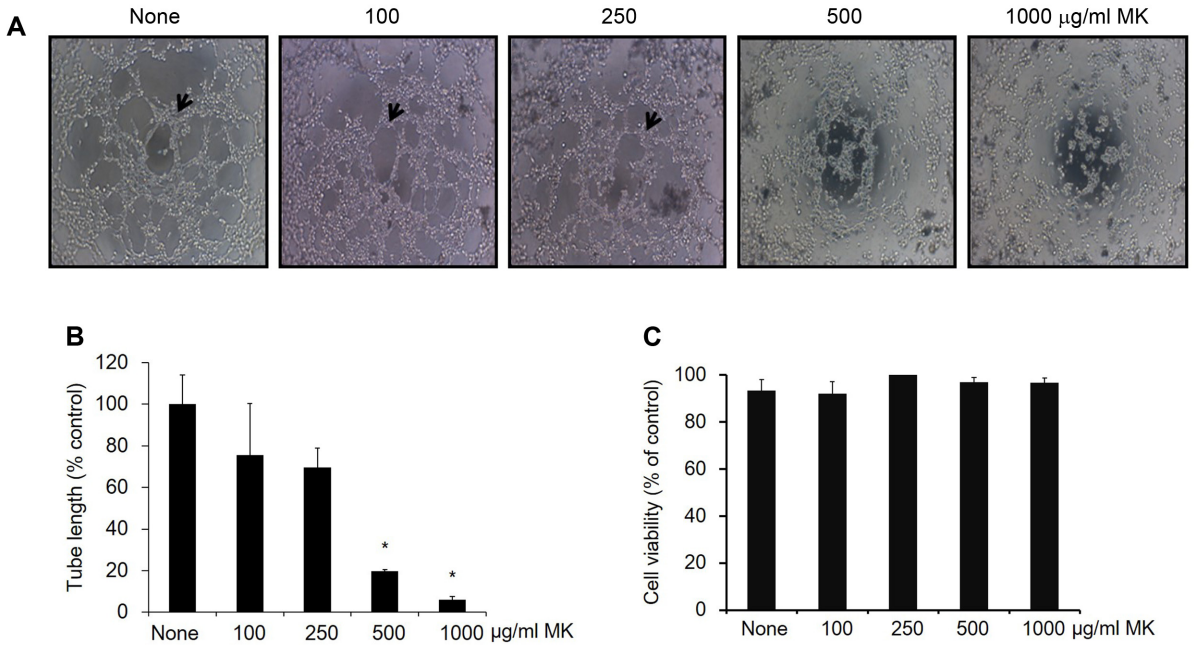

Fig. 1. Effect of MK on tube formation in human umbilical vein endothelial cells (HUVECs).
HUVECs were grown in Matrigel-coated 96-well plates with MK (100, 250, 500, and 1,000 μg/ml) for 20 h, and then tube-like networks were imaged and were indicated by arrows (**A**) and the relative quantification of tube length was measured using pro-plus 6.0 programs (**B**). Under the same conditions, cytotoxicity was measured using the WST-1 assay (**C**). Data are representative of three independent experiments.

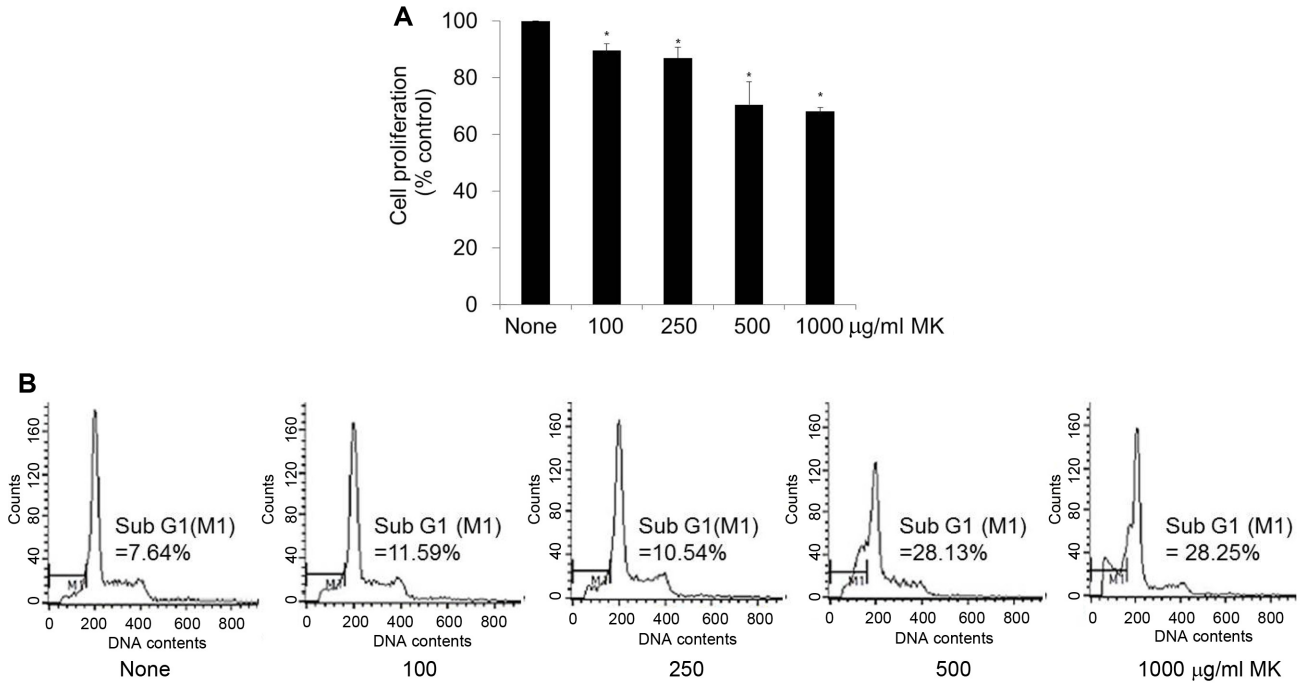

Fig. 2. Effect of MK on cell viability and Sub G1 DNA content in AGS cells.
Cells were treated with MK for 72 h in a dose-dependent manner, and then cell viability was measured by the MTT assay (**A**). Under the same conditions, the Sub-G1 DNA content was measured by PI staining using flow cytometry (**B**). Data are expressed as the mean ± SD. *$p < 0.05$ vs. no treatment.

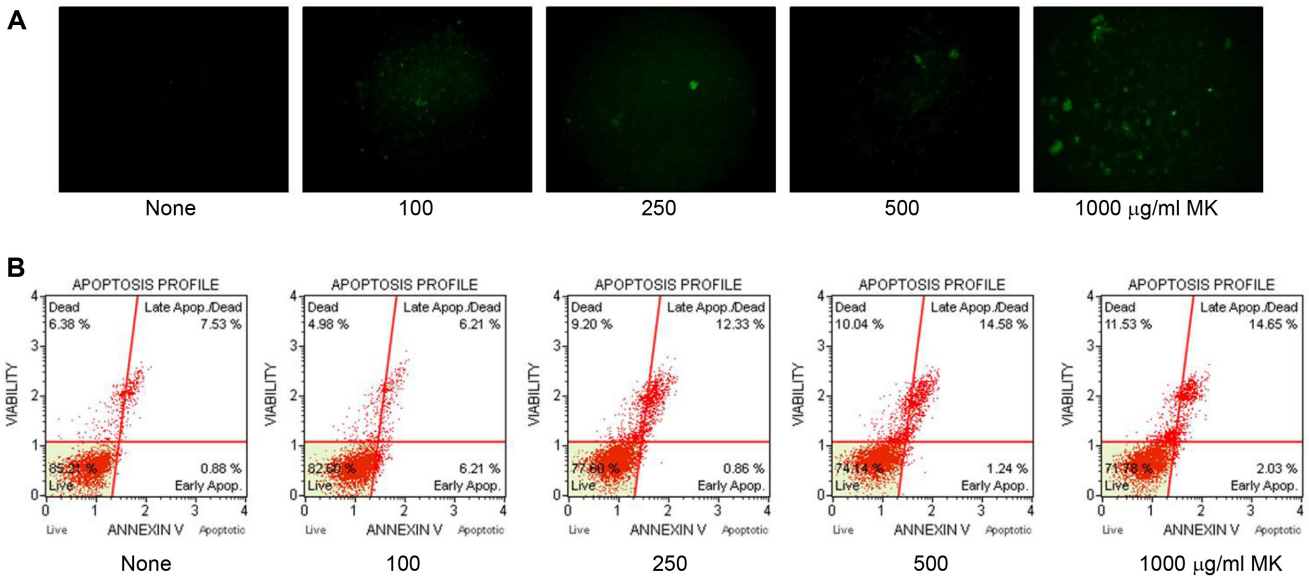

Fig. 3. Effect of MK on terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and Annexin V-positive assay in AGS cells.

Cells were treated with MK for 48 h in a dose-dependent manner, and then cells with labeled 3′-OH ends of fragmented DNA were determined under a fluorescence microscope (Green color dot represents apoptotic cells, ×200) (**A**). Under the same condition, cells were stained with Annexin V and were analyzed by flow cytometry (**B**).

induced by MK in AGS cells. As shown in Fig. 4, MK at concentrations of 250, 500, and 1,000 μg/ml significantly stimulated PTEN expression; under the same conditions, p53 expression was not changed by MK. Taken together, these results demonstrate that MK can induce PTEN in AGS cells.

MK Reduced Cancer Cell Growth in a Tumor Xenograft Model

We finally examined the effects of MK on the growth of AGS cancer cells in a mouse tumor xenograft model. To this end, nude mice were inoculated with AGS cells as described in Materials and Methods. MK was administered orally at 500 mg/kg daily for 7 weeks. As shown in Fig. 5A, the tumor volume and weight of the MK-administered group were significantly reduced compared with the PBS-administered control group. There were no significant differences in body weight between the groups (Fig. 5B), demonstrating that MK exerts an anti-tumor effect without cytotoxicity

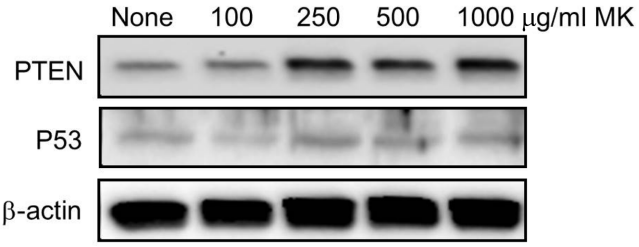

Fig. 4. Effect of MK on phosphatase and tensin homolog (PTEN) and p53 expression in AGS cells.

Cells were treated with MK for 24 h in a dose-dependent manner. PTEN, p53, and beta-actin expression levels were measured by western blot analysis in AGS cells. Data are representative of three independent experiments.

Discussion

In the present study, we have shown that dealcoholized *makgeolli* can inhibit angiogenesis in HUVECs and also induce apoptosis in AGS cells. Furthermore, the pro-apoptotic effect of MK is accompanied by the induction of PTEN expression.

Makgeolli has traditionally been consumed in Korea and is generally brewed by adding *nuruk*, rice, and yeast [8, 9]. Although the importance of the biological activity of *makgeolli* has traditionally been suggested in Korea, thus far, there has been no credible scientific evidence. Therefore, we investigated whether MK exerts an anti-cancer effect in cell culture systems. To this end, we first examined the

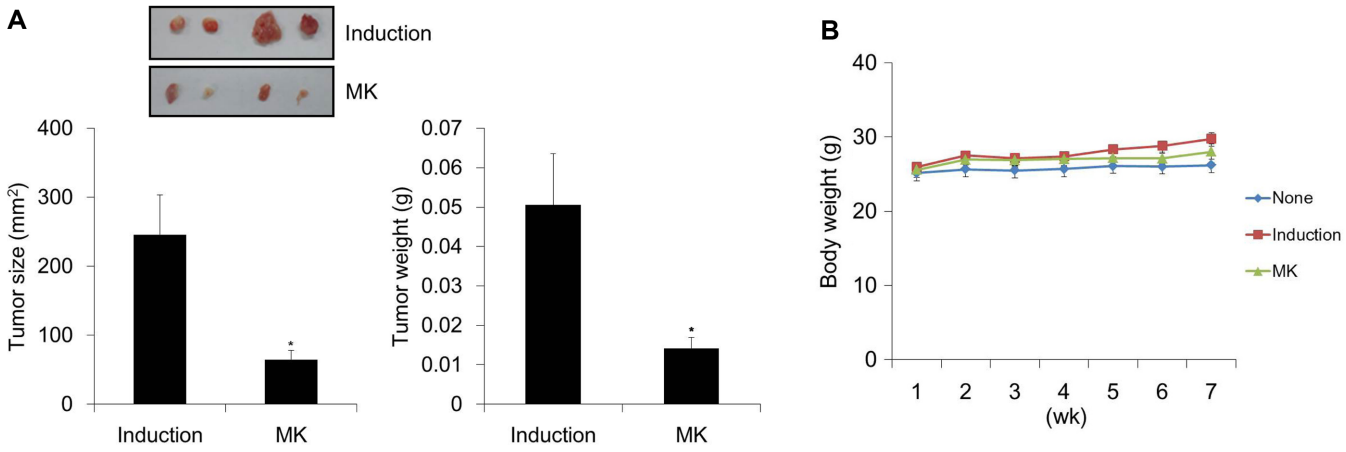

Fig. 5. Decrease in tumor volume due to MK in a tumor xenograft model.
Tumor volume and weight of the MK-administered group were compared with the PBS-administered group (induction) (**A**). Body weight changes between each group (**B**). None, untreated; induction group, AGS cell inoculation; MK, AGS cell inoculation with MK at 500 mg/kg·d. Data are expressed as the mean ± SD (n = 6 per group). $^{*}p < 0.05$ vs. induction.

anti-angiogenic activity of MK by using the tube formation assay, a well-known tool for evaluating anti-angiogenic activity *in vitro* [17]. Angiogenesis is reported to be an important strategy for the survival of cancer cells and is abnormally increased in malignant cancers [18]. To adapt to the tumor microenvironment such as hypoxic conditions, tumor cells induce capillary tube formation by releasing angiogenic factors [18]. Several angiogenic factors, including vascular endothelial growth factor (VEGF) and epidermal growth factor, are produced by progression of angiogenesis and play a critical role in proliferation, migration, and capillary tube formation in cancer cells [18, 22]. It has been reported that plant extracts prevent tube formation *in vitro* and also inhibit hypoxia-induced VEGF expression in various cancer cell types [13, 17]. In this study, we showed that MK significantly inhibited tube formation related to angiogenesis in HUVECs. Therefore, we speculate that the anti-angiogenic effect of MK possibly occurs through the down-regulation of angiogenic factors, including VEGF. On the other hand, *makgeolli* contains various proteins, vitamins, bioactive compounds, and organic acids that are believed to exert beneficial health effects [8, 9]. Several research groups have demonstrated that *makgeolli* exhibits anti-oxidant, anti-hypertensive, anti-diabetes, and anti-cancer effects [8, 9]. More recently, a paper supported the anti-cancer ability of farnesol, a bioactive sesquiterpene alcohol that is involved in *makgeolli* [4]. Farnesol has been reported to have chemopreventive and anti-tumor effects through down-regulation of angiogenic factors, including VEGF [20]. In addition, farnesol induces apoptosis *via* increasing apoptotic proteins in cancer cells [7, 20]. Here, we have shown that MK reduces cancer cell proliferation and increases the sub-G1 population in a dose-dependent manner. Moreover, we found that MK-induced cell death is accompanied by apoptotic motifs in AGS cells, suggesting that MK is effective in inducing apoptosis in AGS cells. Therefore, we suggest that the pro-apoptotic effect of MK is possibly through bioactive compounds such as farnesol, in this study. However, we did not identify precise active compounds that exhibit the pro-apoptotic effect of MK. Further studies are needed to determine the constituents of MK that are most active for the pro-apoptotic effect. Tumor suppressor proteins are important biomarkers for cancer survival and play key roles in repressing cell cycle progression and promoting apoptosis. Tumor suppressor proteins such as retinoblastoma protein, p53 tumor-suppressor protein, and PTEN are well characterized and are crucial targets for chemotherapeutic agents [14, 16]. Among these, p53 has a pivotal role in regulating the cell cycle, and mutated p53 is found in colon, breast, lung, and other cancers [14]. PTEN is a phosphatase of PI3K, which is a critical protein for tumor progression *via* activation of downstream Akt [16]. It is expressed widely in various tissues and dephosphorylates the 3′-phosphate of the inositol ring in phosphatidylinositol (3,4,5)-trisphosphate (PtdIns (3,4,5)P_3 or PIP_3), resulting in inhibition of the AKT signaling pathway [5, 6, 16]. In the present study, MK significantly stimulated PTEN expression in AGS cells; however, p53 expression was not changed by MK. It is well established that interactions between PTEN and p53 induce apoptosis

in response to DNA damage. However, another paper has also shown that PTEN can induce apoptosis independently of p53 in response to DNA damage. Therefore, we speculate that stimulation of PTEN by MK may induce apoptosis *via* p53-independent mechanisms in AGS cells [11, 12].

Earlier reports have stated that plant extracts are potential anti-cancer agents that inhibit cell growth, cell migration, and angiogenesis, and induce apoptosis by induction of PTEN expression in various cancer cells [1, 25–27]. Other studies also demonstrated that honokiol attenuates the angiogenic activities in human endothelial cells and inhibits PI3-K/Akt/mTOR (mammalian target of rapamycin) signaling by reducing Akt phosphorylation and up-regulating PTEN expression in breast cancer [3]. PTEN has also been reported to enhance the pro-apoptotic ability of curcumin, an active ingredient derived from the rhizome of the plant *Curcuma longa* [19]. MK also exerts an anti-cancer effect and induces PTEN expression; this finding is partially consistent with those of previous reports. Therefore, we suggest that MK may be a functional material for preventing gastric cancer development and induction of PTEN. However, how MK influences PTEN signaling pathways for preventing cancer development and which major constituent compounds of MK are responsible for the anti-cancer effects in AGS cells remain unclear. The purpose of this study was to evaluate the anti-cancer activity of MK and demonstrate its molecular mechanism in cancer cells. To the best of our knowledge, this is the first report to suggest that MK prevents *in vitro* angiogenesis and induces apoptosis in AGS cells. In addition, we demonstrated that MK significantly induced the expression of PTEN in AGS cells. Furthermore, MK significantly reduced the tumor volume and size in AGS tumor xenografts. Our findings highlight the anti-cancer effect of MK and facilitate an understanding of the target for preventing diseases by using MK. This study will be a good scientific background for foods containing non-alcoholic *makgeolli*.

Acknowledgments

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