Extract of *Makgeolli Suldeot* Downregulates NF- κ B-mediated MMP-9 Expression, thereby Inhibiting Invasive Activity in a Human Breast Cancer Cell Line, MDA-MB-231

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Abstract *Makgeolli* is a Korean traditional alcoholic beverage prepared by fermenting rice starch using *nuruk*. It is known for its health benefits, but the mechanisms underlying its inhibitory effects on cancer remain unclear. Here a 70% ethanol extract of *Makgeolli suldeot* (70EMS) was tested for its inhibitory activities against cancer metastasis. This extract interrupted the formation of the neovasculature and reduced the migratory and invasive activity of breast cancer cells. Furthermore, it dose-dependently suppressed the activity of matrix metalloproteinase (MMP)-9 and phosphorylation of ERK1/2, JNK, and Akt. Moreover, 70EMS strongly inhibited the translocation of nuclear factor-kappa B (NF- κ B) into the nucleus and expression of activator protein-1 (AP-1). These data indicate that the anti-invasive effect of 70EMS may be related to the inhibition of ERK1/2, phosphorylation of JNK, and reduced expression of NF- κ B and AP-1, thereby attenuating MMP-9 in human breast cancer cells.

Keywords: anti-invasion, breast cancer, *makgeolli suldeot*, matrix metalloproteinase-9, nuclear factor-kappa B

Introduction

Metastasis is defined as the spread of cancer from its primary site to distant metastatic sites and is the cause of death in patients with malignancies. Cancer progression involving invasive and metastatic stages is a seriate process involving the dissociation of malignant cells in the primary tumor, migration, invasion into the vasculature or lymphatic systems, and extravasation in the secondary organs as well as growth of tumor cells in the metastatic organ/tissue (1–3). In these processes, metastatic cells are involved in numerous interactions with the extracellular matrix (ECM), basement membrane, endothelial cell lining, and secondary site microenvironment (3,4). Matrix metalloproteinases (MMPs) play a critical role in ECM component degradation, angiogenesis, tumor invasion, and tumor cell growth and are regarded as major players in cancer progression (4,5). Different tumors may utilize different MMPs. MMPs belong to the family of zinc-dependent endopeptidases that are capable of ECM component degradation (3,6). Proteolytic activation is an important process in the regulation of MMP-dependent activities. MMP-2 and

-9 are critical for motility and invasiveness of tumor cells and are synthesized and secreted as zymogens. Their expression is regulated by the activation of various transcription factors such as activator protein-1 (AP-1) and nuclear factor-kappa B (NF- κ B), which are activated by signal transduction pathways of mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-3 kinase (PI3K)/Akt (7–10). It has been reported that MMP-2 and -9 contribute to invasion, angiogenesis, and metastasis of breast cancer (3,11–13).

Makgeolli is a Korean traditional wine that originated in the 10th century. In contrast to the red wine production process, in which yeasts convert natural grape sugars into alcohol and carbon dioxide, the brewing process for *makgeolli* involves the use of *nuruk* for fermentation. *Makgeolli* is a representative of simultaneous saccharification and alcohol fermentation. *Nuruk* is a starting culture comprising various microorganisms, including lactic acid bacteria (LAB). It is responsible for saccharifying rice starch during the fermentation process (14,15). Although *makgeolli* is brewed in a manner similar to other Korean traditional wines, it is not filtered; therefore, it appears turbid, has an opaque milky color, and contains

6–7% of alcohol. There are two types of *makgeolli*: draught (unpasteurized) and pasteurized. *Makgeolli* differs from other alcoholic beverages in that it has high levels of nutrients, including essential amino acids, proteins, vitamin B, acetylcholine, dietary fiber, and various organic acids (16,17). In particular, draught *makgeolli* contains many residual rice carbohydrates and live microflora such as LAB and yeasts; therefore, it is renowned for its health benefits (14). In addition, draught *makgeolli* has a refreshing taste owing to carbonation by fermentation.

Makgeolli is also abundant in polyphenols such as flavonoids (flavonols), which have strong scavenging activities against the hydroxyl, superoxide anion, and nitric oxide radicals (18). It has been reported that the *makgeolli* extract decreases serum levels of total cholesterol and triglycerides and inhibits the activity of quinine reductase and growth of diverse tumor cells (19,20). However, it is unclear if *makgeolli* and its extracts have inhibitory effects on tumorigenicity. Therefore, in this study, we examined the inhibitory mechanism of invasiveness, particularly with respect to the activation of cell signaling pathways, of the 70% ethanol extract of *makgeolli suldeot* (70EMS) using a human breast cancer cell line.

Materials and Methods

Chemicals The draft *makgeolli suldeot* was kindly provided by Kooksoondang Brewery Co. Ltd. (Seoul, Korea). The dried 70EMS was dissolved in dimethyl sulfoxide (DMSO) as a stock solution (stock concentration: 100 mg/mL in DMSO), stored at 20°C, and diluted with cell culture medium before each experiment. The primary antibodies for MMP-2, MMP-9, p38, phospho-p38, ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, Akt, phospho-Akt, NF-κB, IκB, phospho-IκB, c-Jun, and phospho-c-Jun were obtained from Cell Signaling Technology (Beverly, MA, USA). Phospho-NF-κB (p65), c-Fos, and lamin A/C antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fetal bovine serum (FBS), streptomycin, penicillin, and cell culture media were purchased from Gibco-BRL (Grand Island, NY, USA). Fertilized eggs were purchased from Yangsung Animal Farm (Yongin, Korea). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Cell culture and viability assay The breast cancer cell line MDA-MB-231 was obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI1640 supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 units/mL) and incubated at 37°C in a humidified 5% CO₂ atmosphere. Cell viability was quantified using MTT colorimetric assay. In brief, MDA-MB-231 cells were seeded in a 96-well plate (2×10⁴ cells/well) overnight and treated with 70EMS for 24 h. MTT solution was added to the each well, and the cells were incubated for 4 h at 37°C. The optical density was measured at 540 nm.

Ex vivo CAM assay Fertilized chicken eggs were maintained in a humidified incubator at 37°C and applied to an *ex vivo* CAM assay, as previously described (15). Twenty eggs were used for each concentration of 70EMS. The percentage relative activity was calculated as the number of avascular eggs per total number of viable eggs.

Wound-healing assay Confluent MDA-MB-231 cells (2×10⁵ cells per well in a 24-well plate) were scratched using a 200 μL tip to create a cell-free zone in each well and treated with indicated dosages of 70EMS for 24 h. The image of migrated cells was captured with a Nikon ECLIPSE TE-2000U microscope.

Matrigel invasion assay Cell invasion assays were performed using Transwell chambers coated with Matrigel, following a method previously mentioned (21) with slight modifications. Cells were seeded at a density of 2×10⁵ per chamber in each invasion assay. The cells were plated in the Matrigel-coated upper chambers, and serum-free medium with or without 70EMS was added into the Transwell chambers. The lower chamber was filled with serum-containing medium as a chemoattractant. After 24 h, the invading cells were fixed with 4% (w/v) paraformaldehyde and stained with hematoxylin and eosin. The number of invaded cells was counted at random fields by light microscopy.

Gelatin zymography To determine the expression and activities of MMP-2 and -9 in 70EMS-treated MDA-MB-231 cells, we used gelatin zymography. MDA-MB-231 cells treated with 70EMS were incubated for 24 h, and the cultured medium was collected, concentrated with centricon, and quantified to the protein concentrations. Cultured medium was subjected to SDS-PAGE containing 1 mg/mL gelatin. Subsequently, the gelatin gels were treated with 2.5% (w/v) Triton X-100, incubated in the Zymogram Development Buffer (Bio-Rad, Hercules, CA, USA) and stained with Coomassie Blue R-250. The gelatin gels were destained with water until clear zones appeared.

Western blot analysis MDA-MB-231 cells were lysed in an extraction buffer [25 mM Tris (pH 7.4), 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, 1% Triton X-100, 10 mM NaF, 1 mM Na₃VO₄, and complete protease inhibitor cocktail], and SDS-PAGE was performed. The primary antibodies used were anti-MMP-2, anti-MMP-9, anti-p38, anti-phospho-p38, anti-ERK1/2, anti-phospho-ERK1/2, anti-JNK, anti-phospho-JNK, anti-Akt, anti-phospho-Akt, anti-NF-κB, anti-IκB, anti-phospho-IκB, anti-c-Jun, anti-phospho-c-Jun, anti-phospho-NF-κB (p65), anti-c-Fos, and anti-lamin A/C.

Statistical analysis Student's *t*-test and one-way analysis of variance were used to determine the statistical significance between the values of the various experimental and control groups.

Fig. 1. Inhibitory activities of 70EMS against angiogenesis in the CAM assay. Only photographs of 70EMS-treated CAM samples were selected owing to high relative activity. Dotted-lined circles represent the regions where the coverslips were loaded.

Results and Discussion

70EMS inhibited angiogenesis Angiogenesis is a physiological process that is a critical step in the pathology of tumor progression (22). To investigate whether 70EMS has antiangiogenic properties, we performed a CAM assay. In this assay, 70EMS was loaded on Thermanox coverslips and placed at the vascular membrane of fertilized eggs. Fertilized eggs treated with the indicated concentration of 70EMS survived 17–20 of the 20 eggs. Retinoic acid as a positive control displayed 80% relative inhibitory activity. The inhibitory effects of 70EMS on developmental neovascularization in chick embryos ranged from 33.3 to 82.4% (Fig. 1A and Table 1) compared with those of vehicle-treated control. These results indicate that 70EMS has potential for the inhibition of neovascularization *ex vivo*.

70EMS inhibited tumor cell migration and invasion The human breast cancer cell line MDA-MB-231 was selected for these experiments owing to its high migration potential. We did not observe any overt cytotoxic effect of 70EMS treatment in MDA-MB-231 cells, as assessed by the MTT assay at concentrations up to 50 mg/mL (Fig. 2A).

Tumors can penetrate the basement membrane of tissues by migrating cancerous cells (23). The invasive capacity of tumor cells is required for the multistaged tumor progression (1–3). To examine the inhibitory effect of 70EMS on the migratory potential of MDA-MB-231 cells, we performed a wound-healing assay. As shown in Fig. 2B, the vehicle-treated MDA-MB-231 cells moved into and filled the scratched area to an extent that there was little to distinguish from the wound boundary. In contrast, treatment with 70EMS dose-dependently inhibited the wound-healing activity of MDA-MB-231 cells.

To examine whether 70EMS can inhibit the invasive activity of MDA-MB-231 cells, we performed an invasion assay using a Matrigel-

Table 1. Effect of 70EMS on chick embryonic angiogenesis using CAM assay

Fractions	Final concentration (µg/mL)	No. of viable eggs/No. of total eggs tested	No. of positive eggs	% Relative inhibitory activity ¹⁾
0.1% DMSO ²⁾	10	20/20	1	5.0
Retinoic acid ³⁾	1	20/20	16	80.0
	10	18/20	6	33.3
70EMS	25	18/20	12	66.7
	50	17/20	14	82.4

¹⁾Each assay employed about 20 eggs per sample. Relative activity (%) = (number of positive eggs/number of viable eggs) × 100%

²⁾Unit of 0.1% DMSO: µL

³⁾Unit of retinoic acid: µg

coated Transwell system. The breast cancer cells treated with 70EMS were plated on the Matrigel-coated upper chamber of a Transwell insert. Serum-free medium was added to the upper chamber and a medium containing 10% FBS as a chemoattractant was filled in each well of a cell-culture plate. To quantify the invaded cells, the cells in the opposite side of the Transwell membrane were stained with hematoxylin and eosin and scored and plotted as a percentile. As shown in Fig. 2C, 70EMS reduced the number of invading cells in a dose-dependent manner; 70EMS at concentrations of 10, 25, and 50 mg/mL displayed inhibitory rates of 3.40, 63.9, and 94.9%, respectively (Fig. 2D). These results suggest that 70EMS suppresses cell migration and invasion of MDA-MB-231 cells.

70EMS decreased MMP-9 enzymatic activity and protein expression

To determine whether the inhibitory activities of 70EMS on cancer cell invasion correlated with reduction of the gelatinolytic activities of MMPs, we performed the gelatin zymography and western blot analysis. To do so, MDA-MB-231 cells were treated with 70EMS for 24 h, followed by gelatin zymography. As shown in Fig. 3A, treatment with 70EMS significantly decreased the gelatinolytic activity of MMP-9 but not MMP-2. Consistent with the gelatin zymogram, 70EMS markedly inhibited the expression of MMP-9 in the cell lysate of MDA-MB-231 (Fig. 3B) and the secretion level of MMP-9 (Fig. 3C). Moreover, the secretion level of MMP-9 was almost completely inhibited with 25 mg/mL of 70EMS (Fig. 3C). However, there was no gelatinolytic activity corresponding to the MMP-2 band. Recently, Lee *et al.* (15) reported that NF-κB particularly regulates MMP-9 expression or activity without MMP-2 in cancer cell invasion. For example, capillarasin inhibits NF-κB-dependent MMP-9 expression, resulting in the suppression of PMA-induced tumor cell invasion, and the extract from *Hibiscus sabdariffa* leaf attenuates cell invasion and the activity of MMP-9 through the downregulation of Akt/NF-κB signaling pathway in human prostate cancer cells (23). MMPs have long been functioned as major contributors to the proteolytic degradation of ECM during the invasive process of malignant cells. MMP-9 plays a critical role in various physiological and pathological processes, including

Fig. 2. Inhibitory effect of 70EMS on tumor cell migration and invasion. (A) Cell viability was determined with an MTT assay. MDA-MB-231 cells were seeded at 2×10^4 cells per well in a 96-well plate and treated with 70EMS (10, 25, and 50 mg/mL) for 24 h in serum-free medium. The results are presented as mean $\pm$ SD values of three independent experiments. (B) Cell migration was measured by the wound-healing assay. Cell monolayers were scratched with a pipette tip and then treated with 70EMS for 24 h. Migrating cells were photographed under a phase-contrast microscope. (C) Invasion of MDA-MB-231 cells was measured using Transwell chambers with 6.5-mm diameter polycarbonate filters (8 mm pore size) coated with Matrigel. Photographs are representative of three independent experiments. (D) Inhibition of invasion by 70EMS. The values are mean $\pm$ SD of three independent experiments. *Significantly different from control ($p < 0.01$).

Fig. 3. Inhibitory effect of 70EMS on MMP-9 activity and expression. (A) MDA-MB-231 cells were incubated with 70EMS for 24 h in serum-free medium. MMP-9 activity in the conditioned medium was analyzed by gelatin zymography. (B and C) MDA-MB-231 cells were incubated with 70EMS for 24 h, and MMP-9 and MMP-2 expression in conditioned medium (B) or cell lysates (C) was analyzed with western blot.

angiogenesis and tumor progression (2). The angiogenic switch is essential for, in part, selective remodeling of the tissue's ECM and/or basement membrane before the endothelial cells can sprout and form new blood vessels. A number of proteolytic systems such as MMPs are involved in this tissue remodeling (6,22). Our data indicate that 70EMS suppresses the invasive potential of tumor cells and inhibits the activity and expression of MMP-9, which may underlie its antiangiogenic properties (Fig. 1).

70EMS inhibited ERK1/2, JNK, and Akt phosphorylation To clarify the inhibitory mechanisms underlying the metastatic activity of 70EMS, we determined which cell signaling pathways were affected by the treatment of the breast cancer cells using 70EMS. MDA-MB-231 cells were treated with 10–50 mg/mL of 70EMS for 24 h, and levels of phospho- and total MAPKs and Akt proteins were determined by western blot analysis. As shown in Fig. 4, 70EMS downregulated the phosphorylation of ERK1/2, JNK, and Akt in a dose-dependent manner but did not significantly affect p38 MAPK. The signaling pathways of MAPK and PI3K/Akt play a critical role in regulating the expression of MMPs via transcription factors, including NF- κ B and AP-1 (7,9,10). The MAPK signaling pathways are involved in the regulation of important cellular functions, including proliferation, invasion, and migration, and MMP expression (8,24). In addition, the ERK1/2-dependent expression of MMPs was associated with cell proliferation, invasion, and neovascularization (24). These data suggest that inhibition of tumor cell invasion and neovascularization

Fig. 4. Inhibitory effects of 70EMS on MAPK and AKT phosphorylation. MDA-MB-231 cells were treated with 70EMS at the indicated concentration for 24 h. Total cell lysates were prepared as described in Materials and Methods and subjected to western blot analysis using antibodies against phospho-ERK, phospho-JNK, and phospho-p38. Total levels of ERK, JNK, and p38 were used as loading controls. The results are representative of three independent experiments.

by 70EMS is related to the downregulation of MMP-9 expression, resulting from attenuating the ERK1/2, JNK, and Akt signaling pathways.

70EMS inhibited the MMP-9-related transcription factors NF- κ B and AP-1 The expression of MMP-9 is controlled by the activation of transcription factors such as AP-1 and NF- κ B through PI3K/Akt signaling (9). It has been reported that activation of NF- κ B occurs through the phosphorylation of I κ B α , resulting in the dissociation of NF- κ B subunits, and the activated NF- κ B is translocated from the cytosol into the nucleus where it switches on the expression of specific genes (25). Therefore, we examined whether 70EMS activates NF- κ B. As shown in Fig. 5A, the treatment of MDA-MB-231 cells with 70EMS downregulated the phosphorylation of I κ B α and NF- κ B (p65) in the cytosolic and nuclear fractions, respectively.

Furthermore, the effect of 70EMS on the expression of the transcription factor AP-1 was investigated. MDA-MB-231 cells were treated with 70EMS for 24 h and the nuclear extracts were analyzed by western blot. As shown in Fig. 5B, treatment with 70EMS inhibited the expression level of AP-1 in a dose-dependent manner. c-Jun as a transcriptional factor for MAPK forms homo- and heterodimers in combination with c-Fos to produce the AP-1 transcription complex. Moreover, the AP-1 transcriptional activity depends not only on the abundance but also on the phosphorylation of c-Jun and c-Fos (26). As mentioned above, 70EMS inhibited the phosphorylation of ERK1/

Fig. 5. Inhibitory effect of 70EMS on the phosphorylation of NF- κ B and expression of AP-1. MDA-MB-231 cells were treated with 70EMS. After incubation for 4 h, nuclear extracts and total cell lysates were prepared as described in Materials and Methods. (A) Nuclear extracts and cell lysates were subjected to SDS-PAGE followed by western blot analysis using phospho-NF- κ B (p65) and phospho-I κ B antibodies. Levels of total NF- κ B (p65) and I κ B were used as loading controls. (B) Nuclear extracts were subjected to western blot analysis using phospho-c-Jun, c-Fos, and AP-1 antibodies. Levels of total c-Jun, c-Fos, and lamin A were used as loading controls. The results are representative of three independent experiments.

2 and JNK in a dose-dependent manner. We investigated the effects of 70EMS on the expression of c-Jun and c-Fos and phosphorylation of c-Jun using western blot analysis. Our results showed that the treatment of MDA-MB-231 cells with 70EMS inhibited both phosphorylation and expression of c-Jun and expression of c-Fos in a dose-dependent manner (Fig. 5B). These data suggest that 70EMS suppresses AP-1 activation by inhibiting the ERK/c-Fos and JNK/c-Jun pathways.

In conclusion, we found that 70EMS inhibited breast cancer cell migration, invasion, and angiogenesis *in vitro* with the downregulation of MMP-9 expression. This may be because of the inhibition of Akt, ERK1/2, and JNK phosphorylation and downregulation of NF- κ B and AP-1 expressions of 70EMS. Our results indicate that 70EMS has an anti-invasive effect *in vitro*, which can be useful for *in vivo* analysis of breast cancer metastasis. These findings provide new mechanistic insights into the inhibitory effects of 70EMS on the invasive activity of breast cancer cells and suggest that 70EMS acts as a preventive agent in cancer progression. In further studies, the effects of active compounds isolated from 70EMS with the perspective of development of a novel anticancer therapeutic agent will be elucidated.

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Disclosure The authors declare no conflict of interest.

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