Polysaccharide isolated from the liquid culture broth of *Inonotus obliquus* suppresses invasion of B16-F10 melanoma cells via AKT/NF-κB signaling pathway

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Abstract. A number of polysaccharides exhibit pharmacological activities. Polysaccharides derived from *Inonotus obliquus* (PLIO) appear to have various potential pharmacological properties, including anti-tumor activity. However, the molecular mechanisms underlying these properties remain to be elucidated. The present study investigated the anti-metastatic potential of PLIO and the underlying signaling pathways in B16-F10 murine melanoma cells using the MTT colorimetric assay, *in vitro* migration and invasion assays, and flow cytometric and western blot analyses. PLIO inhibited the invasion of B16-F10 cells and suppressed the expression of matrix metalloproteinases. PLIO treatment inhibited nuclear factor-κB (NF-κB) nuclear translocation in B16-F10 cells. In addition, PLIO treatment inhibited the phosphorylation of c-Jun N-terminal kinases and AKT. These results suggest that PLIO may suppress the invasion of highly metastatic melanoma cells via inhibition of the AKT/NF-κB signaling pathways.

Introduction

Inonotus obliquus has been commonly used in Asian and Russian folk medicine for thousands of years due its health-promoting properties and relatively low toxicity, it has been widely studied in recent years. It has been demonstrated that *I. obliquus*, a fungus in the Hymenochaetaceae family, contain numerous steroids, phenolic compounds and polysaccharides that have various biological activities (1). Numerous mushrooms have biologically active polysaccharides in their fruiting bodies, cultured mycelium, and culture broth. *I. obliquus* has been reported to exhibit various beneficial biological properties, including anti-tumor, anti-metastatic, anti-oxidative, anti-viral, anti-diabetes, and immunomodulatory activities (2-6). The fruiting bodies of wild *I. obliquus* are expensive due to host specificity and rarity in nature. Thus, the production of adequate amounts of the fruiting bodies of wild *I. obliquus* for use as a different chemotherapeutic option is currently impractical. Liquid cultures of mushrooms, in addition to the culture broth and mycelia, are a promising alternative to wild resources for the efficient production of polysaccharides, as this method has high productivity and low cost. It is required to demonstrate that fermented polysaccharides exhibit medicinal and nutritional values that are comparable to those from medicinal mushroom fruiting bodies (7), as previous studies have reported that the mycelial biomass of medicinal mushrooms possesses different pharmacologic properties from those of the mushroom fruiting bodies (8,9). Thus, the present study aimed to investigate the biological activities of polysaccharides from liquid culture with mushroom mycelia.

Cancer is an important cause of human mortality worldwide, and numerous cancer treatments, including cancer chemotherapeutic agents, are known to result in adverse side effects (10,11). Metastasis is a characteristic of highly malignant tumor cells leading to poor clinical outcomes. The excessive degradation of extracellular matrix (ECM) is a characteristic of tumor metastasis and invasion (12). Furthermore, matrix metalloproteinases (MMPs) in humans have been identified as key factors involved in these processes. MMPs are a family of zinc-dependent endopeptidases that facilitate proteolytic cleavage of ECM components, including proteoglycan, collagen, laminin, elastin, and fibronectin (13). It has been demonstrated that the phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) signaling pathways regulate metastasis in various types of

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cancer cell (14). Activation of the PI3K-AKT pathway may increase the expression levels of MMP directly, phosphorylate IκB kinases and activate nuclear factor-κB (NF-κB) signaling pathways, which promote NF-κB translocation to the nucleus and then regulates NF-κB-dependent MMP transcription (15). NF-κB is a key transcription factor in cancer cells, which has been associated with cancer development and maintenance, including preventing apoptosis, growth factor-independent proliferation, and tissue invasion and metastasis (16).

Our previous studies have demonstrated that polysaccharides from the *I. obliquus* fruit body exhibit anti-metastatic activities in B16-F10 murine melanoma cells and A549 human non-small cell lung carcinoma cells (17,18). However, the activities of polysaccharides isolated from liquid cultures of *I. obliquus* (PLIO) remain to be elucidated. Thus, the present study aimed to investigate the anti-metastatic effects and the potential underlying signaling pathways involved in PLIO treatment of the highly metastatic B16-F10 murine melanoma cells *in vitro*.

Materials and methods

Materials. Streptomycin, fetal bovine serum (FBS), and penicillin G and were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Lonza Group, Ltd. (Basel, Switzerland). Isopropyl alcohol and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich. (St. Louis, MO, USA). Antibodies against extracellular signal-regulated kinase (ERK; 1:1,000 dilution; rabbit monoclonal antibody; cat. no. 4695), phosphorylated (p)-ERK (1:1,000 dilution; Thr202/Tyr204 rabbit polyclonal antibody; cat. no. 9101S), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK; 1:1,000 dilution; rabbit polyclonal antibody; cat. no. 9252), p-SAPK/JNK (1:1,000 dilution; Thr183/Tyr185 mouse monoclonal antibody; cat. no. 9255S), p38 MAPK (1:1,000 dilution; rabbit polyclonal antibody; cat. no. 9212), p-p38MAPK (1:1,000 dilution; Thr180/Tyr182 rabbit monoclonal antibody; cat. no. 4631S) and NF-κB p65 (1:1,000 dilution; rabbit polyclonal antibody; cat. no. 3034), and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:2,000 dilution; cat. no. 7074) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). MMP-2 (1:1,000 dilution; rabbit polyclonal antibody; cat. no. 4022), MMP-7 (1:1,000 dilution; rabbit monoclonal antibody; cat. no. 3801), MMP-9 (1:1,000 dilution; rabbit polyclonal antibody; cat. no. 3852), β-actin (1:1,000 dilution; mouse monoclonal antibody; cat. no. sc-47778), and HRP-conjugated goat anti-mouse IgG (1:2,000 dilution; cat. no. sc-2005) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) or BD Biosciences (Franklin Lakes, NJ, USA). All other chemicals were of analytical grade.

Preparation of polysaccharides from the broth of I. obliquus culture. Exopolysaccharide from *I. obliquus* liquid culture broth was isolated using previously described methods (19). Briefly, *I. obliquus* KCTC 26147 was inoculated at 5% (v/v) and cultivated for 7 days at 25˚C, 600 x g, with an uncontrolled pH in a modified medium containing 40 g/l glucose, 5 g/l yeast extract, 1 g/l $MgSO_4 \cdot 7H_2O$, and 2 g/l KH_2PO_4. After 7 days of cultivation, the culture broth was centrifuged at 12,000 x g for 20 min at 4˚C. Polysaccharides were precipitated from the liquid culture broth using 75% ethanol and centrifuged at 8,000 rpm for 20 min. The precipitated polysaccharides were resuspended, dialyzed against distilled water for 3 days to remove low-molecular-weight compounds, and then freeze-dried.

Cell culture. The B16-F10 murine melanoma cell line was obtained from the Korean Cell Line Bank (Seoul, South Korea). Cells were grown in complete DMEM medium supplemented with 10% heat-inactivated FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin. Cells were maintained at 37˚C in a humidified 5% CO_2 incubator.

Cell viability. Cell viability was assessed using the MTT colorimetric assay, as previously described (20). Cells were pre-incubated in 12-well plates for 24 h at 37˚C in a humidified 5% CO_2 incubator. PLIO at different concentrations (1-1,000 μg/ml) was incubated with the cells for 24 h. Following incubation, cells were washed with 1X phosphate-buffered saline (PBS) in order to remove dead cells and 0.5 mg/ml of MTT solution was then added to each well. After 2 h incubation, formazan crystals in each well were dissolved in isopropyl alcohol to solubilize the formazan salt formed. The absorbance was determined at a wavelength of 595 nm using a microplate reader.

Flow cytometry. A fluorescein isothiocyanate (FITC)-labeled Annexin V/propidium iodide (PI) apoptosis detection kit (Molecular Probes; Thermo Fisher Scientific, Inc.) was used to determine the level of apoptosis in tumor cells, according to the manufacturer's protocols. Briefly, cells were harvested using trypsin/EDTA solution, washed with PBS, and centrifuged at 600 x g for 5 min at room temperature to pellet the cells. Cell concentration was adjusted to $1x10^6$ cells/ml and the cells were resuspended in binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, at pH 7.4) prior to staining with FITC-labeled Annexin V and PI for 15 min at room temperature in light-protected conditions. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences) within 1 h after staining. The percentage of apoptotic cells was calculated using the CellQuest software program (version 4.0.4; BD Biosciences). The apoptotic cell rate was calculated as the sum of cells in the early and late phase of apoptosis divided by the total number of events recorded by the flow cytometer.

In vitro migration and invasion assay. Six-well chambers with polycarbonate filters with a pore size of 8.0 μm were used to perform the migration assays. The filters (Corning Incorporated, Corning, NY, USA) were coated with gelatin (Sigma-Aldrich). The cells were seeded to the upper part of the chamber at a density of $1x10^6$ cells/ml with or without PLIO (50 or 100 μg/ml). In the lower chamber, DMEM containing 10% FBS served as a source of chemoattractants. Following incubtion for 24 h, cells that had migrated through the gelatin were stained with 2% crystal violet. The non-migrated cells in the upper chamber were removed with a cotton swab. Images of the migrated cells were captured and the cells were counted under a light microscope (magnification, x40). Cell invasion assays were performed using a Matrigel-coated

Transwell chamber. The cells ($1x10^6$ cells/ml) were seeded to the upper chamber of the Transwell insert with or without PLIO (50 or 100 μg/ml) in serum-free medium. In the lower chamber, DMEM medium containing 10% FBS was used as a source of chemoattractants. Following incubation, cells that had invaded through the Matrigel were fixed with 4% formaldehyde in PBS, stained with 2% crystal violet, images were captured and cells were counted under a light microscope (magnification, x40) (21).

Western blot analysis. Following PLIO treatment (25, 50 or 100 μg/ml), the cells were rinsed with PBS twice and were lysed in lysis buffer [10 mM NaH_2PO_4/$NaHPO_4$ (pH 7.5), 10 mM Tris-HCl (pH 7.5), 1% Triton X-100, 130 mM NaCl, 10 mM NaPPi, 2 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride] on ice for 30 min. The cell lysates were centrifuged of 12,000 x g for 20 min at 4˚C to remove cell debris and the supernatant was collected. Nuclear extracts were prepared using a nuclear extraction kit (Panomics Inc., Fremont, CA, USA) according to the manufacturer's protocol. Protein content was determined using a Bio-Rad Protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal quantities of nuclear and cytosolic protein samples (50 μg per lane) were loaded on 10-15% SDS-PAGE for separation, and transferred onto 0.2 mm Immun-Blot nitrocellulose membranes (Bio-Rad Laboratories, Inc.) by electroblotting. The blot was blocked with 1.5% non-fat milk in 1X Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h, followed by incubation with the specific primary antibodies at 4˚C overnight. The blot was finally incubated with HRP-conjugated secondary antibodies. The membranes were washed with TBS-T after each antibody binding reaction. Detection of protein-antibody complexes was conducted using an enhanced chemiluminescence kit (EMD Millipore, Billerica, MA, USA) followed by exposure to X-ray film.

Statistical analysis. All measurements were from at least three independent experiments and all results are presented as the mean ± standard error of the mean. The data were analyzed using Student's t-test or nonparametric analysis of variance Duncan's multiple range tests were performed to compare multiple groups when appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Treatment with PLIO at low concentrations had no effect on cell viability in melanoma cells. The effect of PLIO on the viability of murine melanoma cell B16-F10 was determined using the MTT assay. Various concentrations (0-1,000 μg/ml) of PLIO were added to the cells followed by incubation for 24 h. Cell viability was determined to be 89% at 200 μg/ml PLIO compared with the control (Fig. 1). These results indicated that PLIO at lower concentrations (0 to 200 μg/ml) did not affect cell viability.

Low concentrations of PLIO did not induce apoptosis of melanoma cells. To determine whether PLIO induces cellular apoptosis in melanoma cells, FITC-labeled Annexin V and PI nucleic acid binding dye were used. Following staining of the cell population with the double staining method, apoptotic cells exhibit green fluorescence, dead cells exhibit red and green fluorescence, and live cells exhbit little or no fluorescence (22). Apoptotic cells were detected by flow cytometry. Low concentrations of PLIO (25, 50, or 100 μg/ml) did not increase apoptosis (Fig. 2A and B), suggesting that PLIO, at ≤100 μg/ml, did not induce cell death or apoptosis in B16-F10 cells. This concentration range was then used in all subsequent experiments.

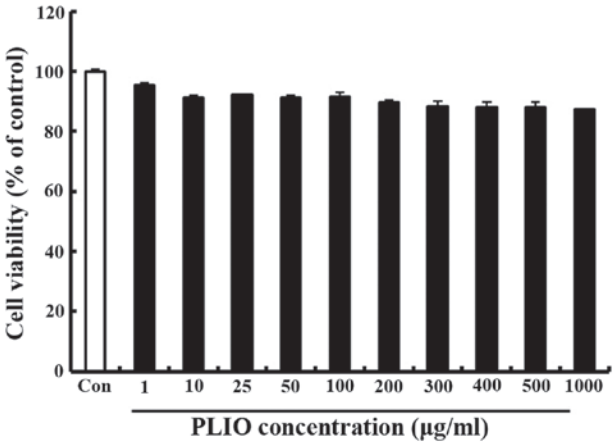

Figure 1. Effect of PLIO on viability of B16-F10 cells. Cells were treated with the indicated concentrations of PLIO for 24 h. Cell viability was measured by the MTT assay. The results are presented as the mean ± standard error of the mean. PLIO, polysaccharides derived from *I. obliquus*.

PLIO did not affect migration of B16-F10 cells, however, invasion of the melanoma cells was inhibited. To investigate whether PLIO had *in vitro* anti-metastatic activity, the present study evaluated B16-F10 cell migration and invasion in the presence of PLIO using gelatin- or Matrigel-coated Transwell assays with polycarbonate filters (pore size, 8-μm). It was observed that B16-F10 cells migrated from the upper chamber to the lower chamber in the untreated control, suggesting that the cells are able to migrate through a gelatin-coated Transwell insert. PLIO at concentrations of 50 and 100 μg/ml did not inhibit B16-F10 cell migration, which was 97 and 92% of the control level, respectively (Fig. 3). In the results of the invasion assay, untreated B16-F10 cells moved from the upper chamber to the lower chamber, indicating that the melanoma cells can invade through the Transwell insert pre-coated with Matrigel (Fig. 4). However, the presence of PLIO had an inhibitory effect on the invasion of B16-F10 cells in a concentration-dependent manner. As presented in Fig. 4B, 100 μg/ml of PLIO significantly inhibited invasion of B16-F10 melanoma cell to 35% ($P<0.05$). Thus, PLIO could inhibit invasion of melanoma cells.

PLIO regulated the expression of MMP-2, MMP-7 and MMP-9 in melanoma cells. ECM degradation, which is key in cellular invasion, involves matrix-degrading proteinases, including MMPs. To determine whether PLIO suppressed MMP protein expression levels, western blotting was used. PLIO treatment decreased the expression of MMP-2 and MMP-9 in B16-F10 cells. Particularly, PLIO was observed to reduce the expression levels of MMP-7 (Fig. 5). These results suggest that PLIO regulated the expression level of MMPs.

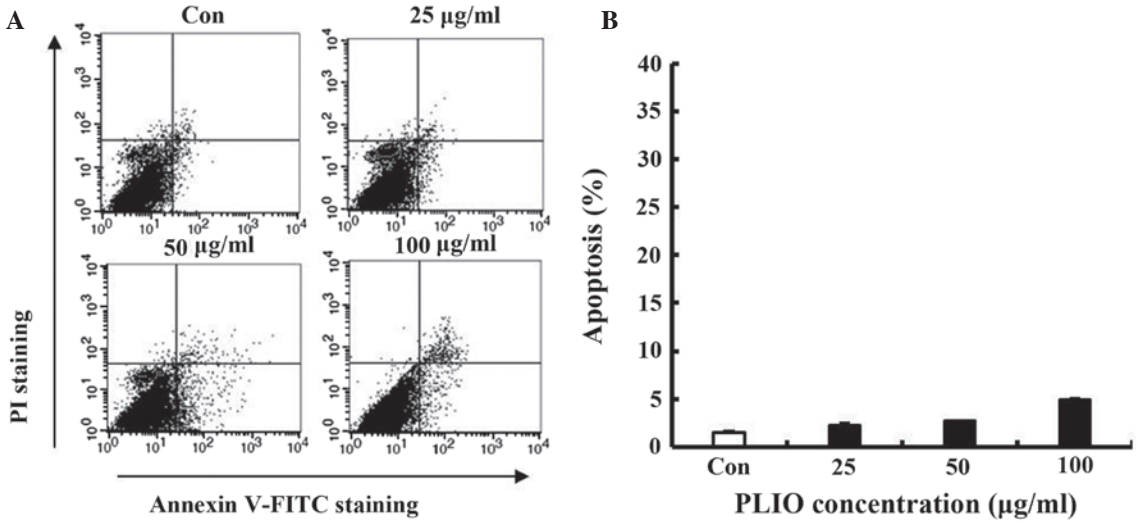

Figure 2. Effects of PLIO on apoptosis of B16-F10 cells. Cells were treated with the indicated concentrations of PLIO for 24 h. (A) Percentage of apoptotic cells after the treatment of PLIO in B16-F10 cells were measured by the Annexin V/PI flow cytometric analysis. (B) Apoptotic index (%) was calculated as the sum of early and late apoptotic cells divided by the total number of events. Con, control; PI, propidium iodide; FITC, fluorescein isothiocyanate; PLIO, polysaccharides derived from *I. obliquus*.

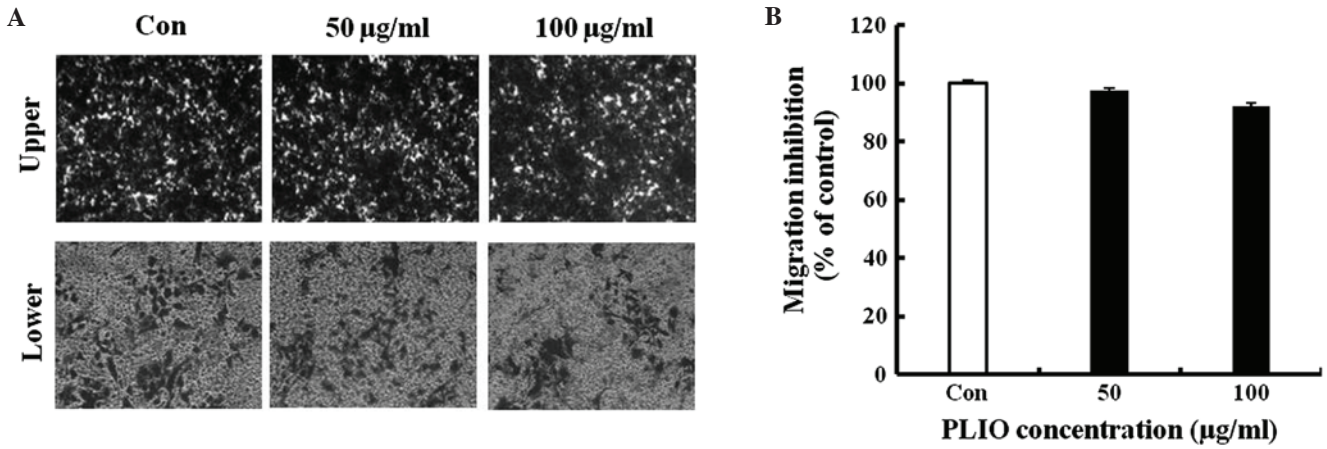

Figure 3. Effects of PLIO on the migration of B16-F10 cells. Cells were treated with the indicated concentrations of PLIO for 24 h. (A) Cell migration assays were performed using gelatin-coated Transwell chambers. (B) Cells that migrated through the gelatin were stained with 2% crystal violet, images were captured and the cells were counted under a light microscope (magnification, x40). PLIO, polysaccharides derived from *I. obliquus*; Con, control.

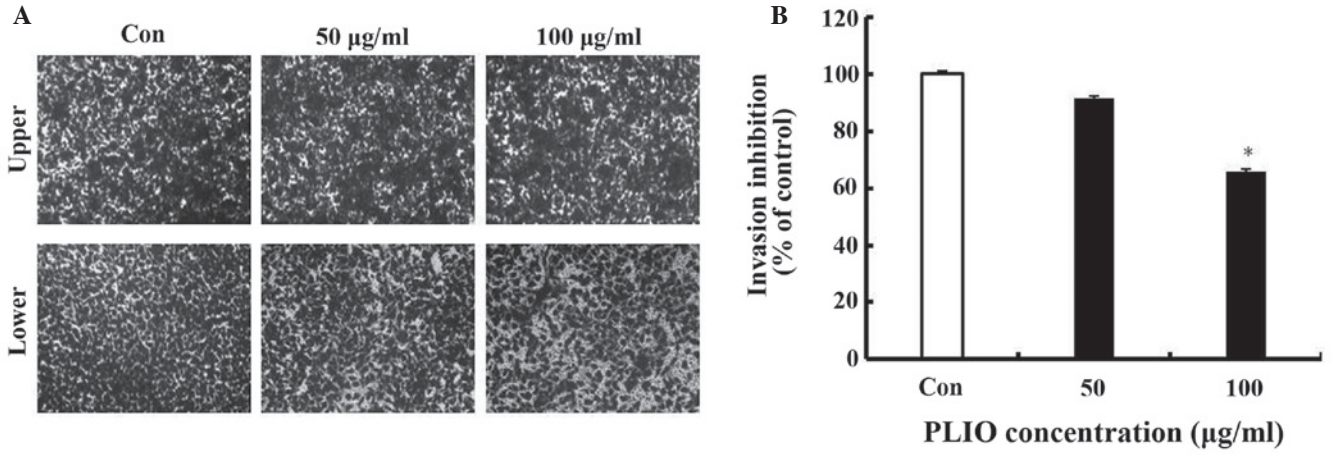

Figure 4. Effect of PLIO on the invasiveness of B16-F10 cells. Cells were treated with the indicated concentrations of PLIO for 24 h. (A) Cell invasion assays were performed using Matrigel-coated Transwell chambers. (B) Cells that invaded through the Matrigel were stained with 2% crystal violet, images were captuted and cells were counted under a light microscope (magnification, x40). *P<0.05 vs. the control group. PLIO, polysaccharides derived from *I. obliquus*; Con, control.

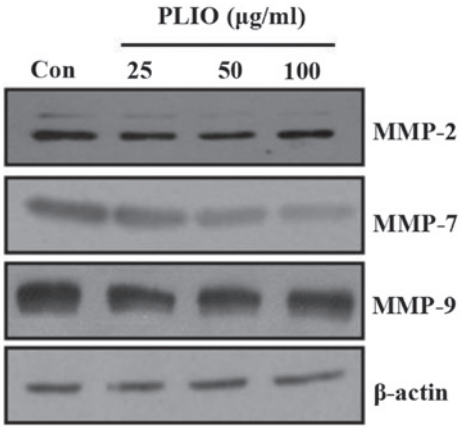

Figure 5. Effect of PLIO on the expression of MMPs in B16-F10 cells. Cells were treated with the indicated concentrations of PLIO in serum-free medium for 24 h. Western blot analysis was used to determine the expression levels of MMP-2, MMP-7, and MMP-9. PLIO, polysaccharides derived from *I. obliquus*; Con, control; MMP, matrix metalloproteinase.

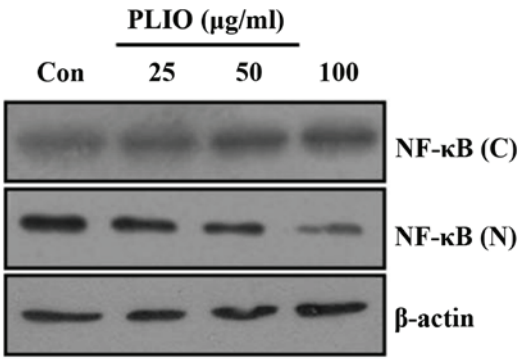

Figure 6. Effect of PLIO on NF-κB translocation in B16-F10 cells. Cells were treated with the indicated concentrations of PLIO for 24 h. The nuclear (N) and cytosolic (C) fractions from the PLIO-treated cells were isolated and analyzed for the expression of NF-κB using western blot analysis. PLIO, polysaccharides derived from *I. obliquus*; Con, control; NF-κB, nuclear factor-κB.

PLIO inhibited NF-κB nuclear translocation in melanoma cells. To investigate whether PLIO inhibits the activation of the NF-κB signaling pathway in melanoma cells, the current investigated the effects of PLIO on translocation of the NF-κB protein from the cytoplasm to the nucleus. Western blotting was used to determine the levels of NF-κB translocation. Cytosolic protein levels of NF-κB in B16-F10 cells were higher in PLIO-treated cells than those in untreated cells (Fig. 6). By contrast, PLIO treatment markedly decreased nuclear protein levels of NF-κB compared with the levels in the untreated control. These results suggest that PLIO inhibited the activation of NF-κB in melanoma cells.

PLIO inhibited phosphorylation of JNK and AKT in melanoma cells. A previous study demonstrated that the activation of the PI3K/AKT and MAPK signaling pathways promotes cancer cell invasion and migration (23). It was demonstrated that PI3K/AKT and MAPK signaling pathways in different tumor cell types may be partially responsible for induction of MMP expression (14,16,24). To investigate whether PLIO regulates PI3K/AKT and MAPK signaling pathways in melanoma cells,

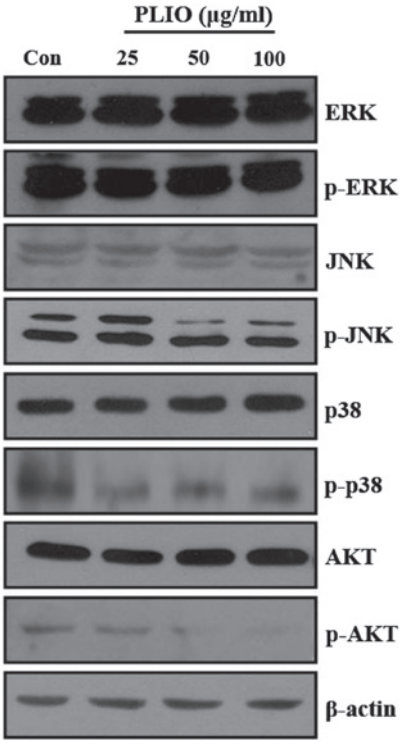

Figure 7. Effect of PLIO on AKT and MAPK pathways in B16-F10 cells. Cells were treated with the indicated concentrations of PLIO for 24 h. The expression and phosphorylation levels of ERK, JNK, p38 MAPK, and AKT were assessed by western blot analysis. PLIO, polysaccharides derived from *I. obliquus*; Con, control; p, phosphorylated; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinases.

the levels of p-ERK, p-p38 MAPK, p-JNK, and p-AKT protein in B16-F10 cells were evaluated by western blot analysis following PLIO treatment. The western blotting demonstrated that the protein expression levels of PI3K/AKT and MAPK was not affected by PLIO treatment, however, the phosphorylation levels of JNK and AKT were inhibited by the addition of PLIO at 50 and 100 μg/ml (Fig. 7). This suggests PLIO inhibited the phosphorylation of JNK and AKT in B16-F10 cells.

Discussion

Research has recently focused on the anti-tumor properties of natural components for their potential chemotherapeutic applications. Polysaccharides are often associated with notable pharmacological activities. For example, polysaccharides extracted from mushrooms, including *Agaricus blazei*, *Phellinus linteus*, *Hericium erinaceus*, and *I. obliquus* exhibit important pharmacological properties. Tumor metastasis is a multi-step process, with complex regulation, which includes angiogenesis, cell attachment, adhesion, migration, invasion, and cell proliferation (25,26). Developing therapeutic agents that inhibit metastasis is considered key, however, effective anti-metastatic agents require further research. The extracellular matrix and basement membrane are stable structures that provide organizational structure. MMPs, which are important for the degradation of extracellular matrix and basement membrane, have been extensively studied and their expression demonstrated to be markedly increased in a variety of types of cancer. In addition, MMPs promote cancer growth

by activating cancer tissue growth factors and inhibiting the apoptosis of cancer cells. MMPs are key in physiological and pathological matrix turnover. Numerous reports have indicated that the expression levels of MMP-1, -2, -7, -9, and -10 are notably increased during cancer cell invasion, and may serve as independent prognostic factors for unfavorable prognosis (14,24). MMP-7 expression in primary melanomas and in metastatic melanoma has been associated with melanoma progression and cell invasion (27). The present study investigated whether PLIO suppressed melanoma cell migration and invasion *in vitro*. The results demonstrated that PLIO suppressed the invasive ability of B16-F10 melanoma cells and suppressed the expression of MMPs in B16-F10 cells. Previous reports have demonstrated that the activation of activator protein 1 and NF-κB via multiple signaling pathways may induce transcription of MMPs and enhance the invasion of tumor cells (28,29). In the present study, PLIO suppressed the expression of MMPs and inhibited the translocation of NF-κB from the cytosol to the nucleus in melanoma cells. These results indicate that PLIO inhibits the metastasis of B16-F10 cells by suppressing the expression of MMPs via inhibiting the NF-κB signaling pathway. It was reported that COX-2, one of the downstream targets of NF-κB, is important in angiogenesis, invasion and migration. It has also been demonstrated to modulate the expression of MMPs (30). The way in which MMPs are regulated by upstream factors, including COX-2, has been investigated in previous studies (31,32). Although multiple genetic alterations are required in cancer invasion and metastasis, COX-2 is involved in the progression of cancer and may be useful in the development of targeted therapies in cancer cells. The current study demonstrated that protein expression levels of COX-2 in B16-F10 cells treated with PLIO were lower than those in the untreated cells, although this difference was not indicated to be statistically significant (data not shown). PLIO may also regulate the invasion of B16-F10 melanoma cells via different mechanisms, including PI3K/AKT and MAPK signaling pathways. It is generally demonstrated that the PI3K/AKT and MAPK signaling pathways regulate metastasis in a variety of cancer cells.

In conclusion, inhibition of metastasis is a key issue in cancer research. PLIO may inhibit the invasion of highly invasive melanoma cells by inhibiting MMPs expression via downregulation of the NF-κB, AKT, and/or MAPK signaling pathways. Based on these findings, the exact underlying anti-metastatic mechanism of PLIO is remains unclear, however, it is concluded that PLIO exhibits potent anti-metastatic effects.

Acknowledgements

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