Polysaccharide from *Inonotus obliquus* inhibits migration and invasion in B16-F10 cells by suppressing MMP-2 and MMP-9 via downregulation of NF-κB signaling pathway

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Abstract. Polysaccharides derived from *Inonotus obliquus* (PIO) are known to possess multiple pharmacological activities including antitumor activity. However, the possible molecular mechanisms of these activities are unknown. In the present study, we determined the anti-metastatic potential and signaling pathways of PIO in the highly metastatic B16-F10 mouse melanoma cell line *in vitro*. We found that PIO suppressed the migration and invasive ability of B16-F10 cells and decreased the expression levels and activities of matrix metalloproteinase (MMP)-2 and MMP-9. In addition, PIO decreased the phosphorylation levels of extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK); PIO also decreased the expression level of cyclooxygenase (COX)-2 and inhibited the nuclear translocation of nuclear factor κB (NF-κB) in B16-F10 melanoma cells. These results suggest that PIO could suppress the invasion and migration of B16-F10 melanoma cells by reducing the expression levels and activities of MMP-2 and MMP-9 through suppressing MAPK, COX-2 and NF-κB signaling pathways.

Introduction

Cancer is a major cause of human mortality worldwide. Many anticancer therapies, including chemotherapy and anticancer drugs, are known to cause adverse side-effects (1,2). Metastatic melanoma accounts for ~80% of melanoma deaths owing to its aggressiveness and resistance to existing therapies (3,4). Metastasis is a characteristic of highly malignant cancers with poor clinical outcomes. Excess extracellular matrix (ECM) degradation is a characteristic of tumor invasion and metastasis (5-7). Matrix metalloproteinases (MMPs) aid tumor cells in MMP degradation. MMPs are a group of zinc-dependent ECM degrading enzymes that facilitate the proteolysis of ECM proteins such as collagen, proteoglycan, fibronectin, elastin and laminin (8). The expression of MMP genes is primarily regulated at the transcriptional level [through AP-1 or nuclear factor κB (NF-κB) via mitogen-activated protein kinase (MAPK) or phosphoinositide 3-kinase (PI3K)/Akt pathways] and post-transcriptional level, as well as at the protein level via their activators or inhibitors and by their cell surface localization (9-12). Recent reports on cyclooxygenase (COX)-2 expression in cancer indicated that this enzyme stimulates tumor growth, invasion and metastasis in association with MMPs (13). COX-2 is an inducible isoform that participates in pro-inflammatory responses to certain stimuli such as mitogens, cytokines and growth factors (14). These studies revealed that MMPs and their regulatory pathways may be promising targets for anti-metastatic and chemotherapeutic therapy.

Mushrooms have been used to treat various diseases including tumors. *Inonotus obliquus*, a traditional medicinal mushroom, has been widely used to promote health and longevity. Many studies reported that *I. obliquus* has many biological activities including antitumor, antimutagenic, anti-oxidative, antimitotic, antihyperglycemic, anti-inflammatory and immunostimulating activities (15-20). However, the anti-metastatic effect and signaling pathway mechanism of polysaccharides from *I. obliquus* (PIO) remain unknown. Therefore, in the present study, we investigated the anti-metastatic effects and potential signaling pathways of PIO in the highly metastatic B16-F10 mouse melanoma cells *in vitro*.

Materials and methods

Preparation of polysaccharides from I. obliquus (PIO). Dried fruiting bodies of *I. obliquus* were purchased from a local market and ground in a blender. Milled mushroom (20 g) was

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Abbreviations: ECM, extracellular matrix; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor κB; COX, cyclooxygenase; Akt, protein kinase B

Key words: Inonotus obliquus, B16-F10 melanoma cell, migration, invasion, MMPs, NF-κB

extracted with distilled water (600 ml) at 121˚C for 2 h. Extracts were centrifuged at 5,000 rpm for 20 min, filtered through 0.45-μm Whatman filter paper (#4; Whatman, UK) to remove insoluble matter and then freeze-dried. Polysaccharides were precipitated from resuspended extracts using 75% ethanol, collected by filtration through 0.45-μm Whatman filter paper, resuspended, and dialyzed against distilled water for 5 days to remove low-molecular-weight compounds (21,22).

Materials. Fetal bovine serum (FBS), penicillin G, and streptomycin were obtained from Gibco (Grand Island, NY, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Lonza (Walkersville, MD, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and isopropyl alcohol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). β-actin monoclonal antibody (mAb), extracellular signal-regulated kinase (ERK) MAPK Ab, phospho-ERK MAPK Ab, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) Ab, phospho-SAPK/JNK Ab, p38 MAPK Ab, phospho-p38 MAPK Ab, COX-2 Ab, MMP-2 Ab, MMP-9 Ab, and NF-κB p65 mAb were purchased from Cell Signaling Technology (Beverly, MA, USA), Santa Cruz Biotechnology (Santa Cruz, CA, USA), or BD Biosciences (San Jose, CA, USA), respectively. All other chemicals were of analytical grade.

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

Cell viability. The effect of PIO on the viability of B16-F10 cells was measured using the MTT assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in viable cells. Cells were pre-incubated in 12-well plates for 24 h at 37˚C humidified atmosphere of 5% CO_2 incubator. Then, cells were incubated with PIO (1-1,000 μg/ml) for 24 h. After incubation, cells were washed with 1X PBS to remove dead cells, and then 50 μl of MTT stock solution (2 mg/ml) was added to each well, which was then incubated for 2 h. After incubation, the MTT assay was performed to quantitate cellular viability. Finally, isopropyl alcohol was added to solubilize the formazan salt formed, and the amount of formazan salt was determined by measuring the absorbance at 595 nm using an enzyme-linked immunosorbent assay microplate reader.

Flow cytometry. The apoptotic death of tumor cells was examined using a fluorescein isothiocyanate (FITC)-labeled Annexin V/propidium iodide (PI) apoptosis detection kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Briefly, cells were harvested by trypsinization, washed with PBS and centrifuged to collect the cell pellet. The number of cells was adjusted to $1x10^6$ cells/ml. The cells were then resuspended in binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, at pH 7.4) and stained with FITC-labeled Annexin V and PI at room temperature for 15 min in the dark. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton-Dickinson, Mountain View, CA, USA) within 1 h after supravital staining. FITC-labeled Annexin V was analyzed using excitation and emission settings of 488 and 535 nm, respectively. PI was analyzed using excitation and emission settings of 488 and 575 nm, respectively. For each flow cytometer run, 10,000 cells were required. The percentages of cells were calculated using the CellQuest software (Becton-Dickinson). The cells in the early stages of apoptosis were Annexin V-positive and PI-negative; however, the cells in the late stages of apoptosis were both Annexin V- and PI-positive. The apoptotic index (%) was calculated as the sum of late apoptotic cells divided for the total number of events.

Wound healing assay. The wound healing assay was performed as previously described with some modifications (23). Briefly, B16-F10 cells were grown to confluence in 6-well dishes for 24 h in serum-free medium (three dishes per group). The medium was replaced with serum-containing medium followed by the addition of PIO at various concentrations (0, 25, 50 and 100 μg/ml), and the cells in monolayer were disrupted (i.e., wounded) by scraping them with a 100-μl micropipette tip. At the indicated times (0, 24 and 48 h) after scraping, the cells were washed twice with PBS (pH 7.4). Finally, the cells were gently washed three times with PBS and photographed with an optical microscope at x40.

In vitro migration and invasion assay. The migration of B16-F10 cells was also measured by chemotactic directional migration using a 6-well Transwell insert. The 8-μm pore filters (Corning Incorporated, Corning, NY, USA) were coated with gelatin (Sigma) and B16-F10 cells ($1x10^6$ cells/ml) were placed in the upper chamber with or without PIO (50 or 100 μg/ml) and allowed to undergo migration for 24 h. The non-migrated cells in the upper chamber were removed with a cotton swab. The filters were stained with 2% crystal violet. Migrated cells adherent to the underside of the filter were counted and photographed using an optical microscope at x40. The invasion of B16-F10 cells was measured using Matrigel-coated Transwell cell culture chambers (8-μm pore size) as previously described. After the cells were cultured for 24 h in serum-free DMEM, they were collected, resuspended in serum-free medium, placed in the upper chamber of the Transwell insert ($1x10^6$ cells/ml), and incubated with or without PIO (50 or 100 μg/ml). DMEM containing 10% FBS was placed in the lower chamber. All the cells in each treatment group were incubated for 24 h at 37˚C in a humidified atmosphere with 95% air and 5% CO_2. The non-invasive cells that remained in the upper chamber were removed by wiping with a cotton swab, and the invasive cells were fixed with 4% formaldehyde in PBS and stained with 2% crystal violet in 2% ethanol. The invasive cells in the lower surface of the filter that penetrated through the Matrigel were counted and photographed using an optical microscope at x40 (24).

Zymography analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) substrate-embedded enzymography (zymography) was used to identify collagenase and gelatinase activities (25,26). Briefly, the supernatant collected from the cell culture was resolved in 10% SDS-PAGE gels,

which were prepared by the incorporation of gelatin (1 mg/ml) before casting. After electrophoresis, the gels were washed twice for 30 min in 2.5% Triton X-100 with shaking. The gels were then incubated at 37˚C for 24-48 h in reaction buffer containing 50 mM Tris-HCl (pH 7.6), 10 mM $CaCl_2$, 150 mM NaCl, and 20% sodium azide, followed by staining with 0.25% Coomassie brilliant blue G-250 in 50% methanol and 10% acetic acid for 1-2 h. The completely stained gels were appropriately destained with 40% methanol and 10% acetic acid. The enzyme activities were evident as clear (unstained) regions against the dark background.

Western blot analysis. After treatment, the cells were washed in 1X PBS and lysed in lysis buffer [10 mM Tris-HCl (pH 7.5), 10 mM NaH_2PO_4/$NaHPO_4$ (pH 7.5), 130 mM NaCl, 1% Triton X-100, 10 mM NaPPi, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A] for 30 min on ice. Lysates were centrifuged at 12,000 x g for 20 min at 4˚C. The supernatant was collected, and its protein content was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) before analysis. The cytosolic or nuclear protein samples were loaded at 10 μg of protein/lane, separated by SDS-PAGE in 10-15% gel, and transferred to NC membranes (Immun-Blot NC membrane, 0.2 μm; Bio-Rad Laboratories). Membranes were blocked with 1.5% skim milk in 1X Tris-buffered saline (TBS) containing 0.1% Tween-20 for 1 h, and they were incubated with primary antibodies at 4˚C overnight. Finally, the membranes were treated with horseradish peroxidase-coupled secondary antibodies for 1 h at 4˚C. The membranes were washed with TBS after each antibody binding reaction. The detection of each protein was performed using an enhanced chemiluminescence kit (Millipore Co., Billerica, MA, USA).

Nuclear protein extraction. Nuclear extracts were prepared by lysing nuclei in high-salt buffer supplemented with protease and phosphatase inhibitors using a nuclear extraction kit (Panomics Inc., Fremont, CA, USA) according to the manufacturer's protocol. Protein concentrations were quantified using the Bio-Rad protein assay.

Statistical analysis. Data are expressed as mean ± standard error (SE) values, and the results were obtained from at least three independent experiments performed in triplicate. The data were analyzed using Student's t-test to evaluate significant differences. A P<0.05 was regarded as statistically significant.

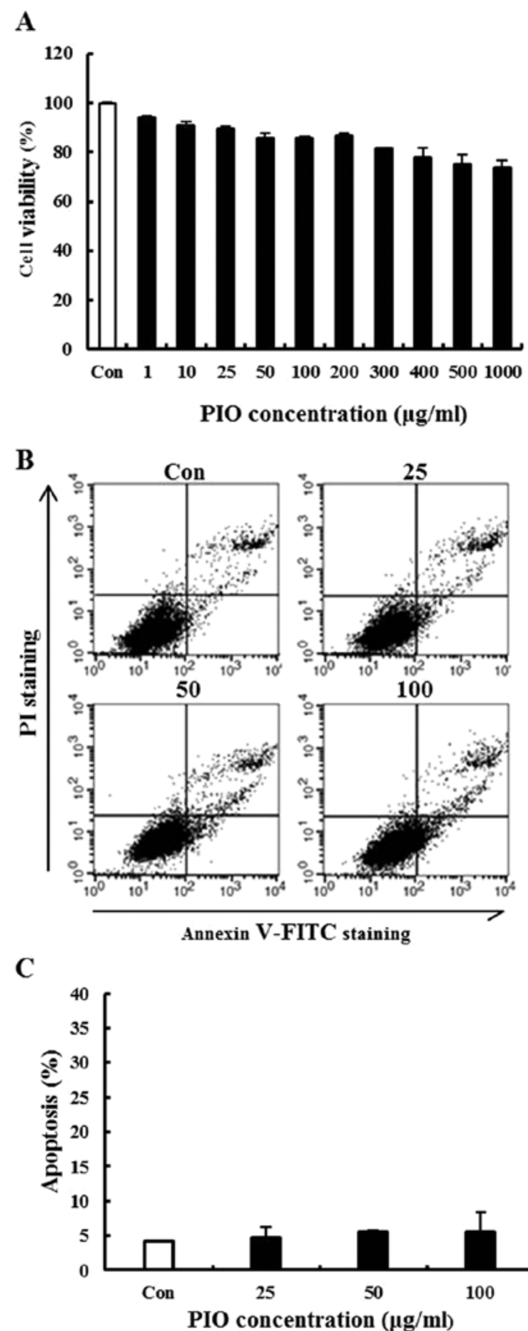

Figure 1. Effect of PIO on cell viability and apoptosis in the B16-F10 cells. (A) The B16-F10 cells were incubated with various concentrations (0-1,000 μg/ml) of PIO for 24 h. (B and C) B16-F10 cells were incubated with various concentrations (0-100 μg/ml) of PIO for 24 h. The number and percentage of apoptotic cells was detected by Annexin V/PI flow cytometric analysis. The data represent the means ± SE of three independent experiments.

Results

Effect of PIO on viability in B16-F10 cells. To investigate the cytotoxicity of PIO in B16-F10 melanoma cells, cells were treated with PIO at various concentrations ranging from 0 to 1,000 μg/ml for 24 h and cell viability was determined by the MTT assay. We found that PIO did not significantly affect the growth of B16-F10 cells (Fig. 1A). To evaluate whether the growth-inhibitory of PIO was associated with apoptosis, a double-staining method using FITC-labeled Annexin V and PI was performed. During the early stage of apoptosis, cells display phosphatidylserine on their outer cell membranes, which is readily detectable using Annexin V. During the later stages of apoptosis, as the plasma membrane becomes increasingly permeable, PI can move across the cell membrane to bind to cellular DNA. Double staining the cells with Annexin V and PI allowed us to detect apoptotic cells by flow cytometry. Low concentrations of PIO (25, 50 or 100 μg/ml) did not have an apoptotic effect on the cells (Fig. 1B and C). Therefore, these results demonstrated that PIO at concentrations ranging from 0 to 100 μg/ml did not induce cell death and apoptosis in the highly metastatic B16-F10 melanoma cell line. This concentration range was then applied in all subsequent experiments.

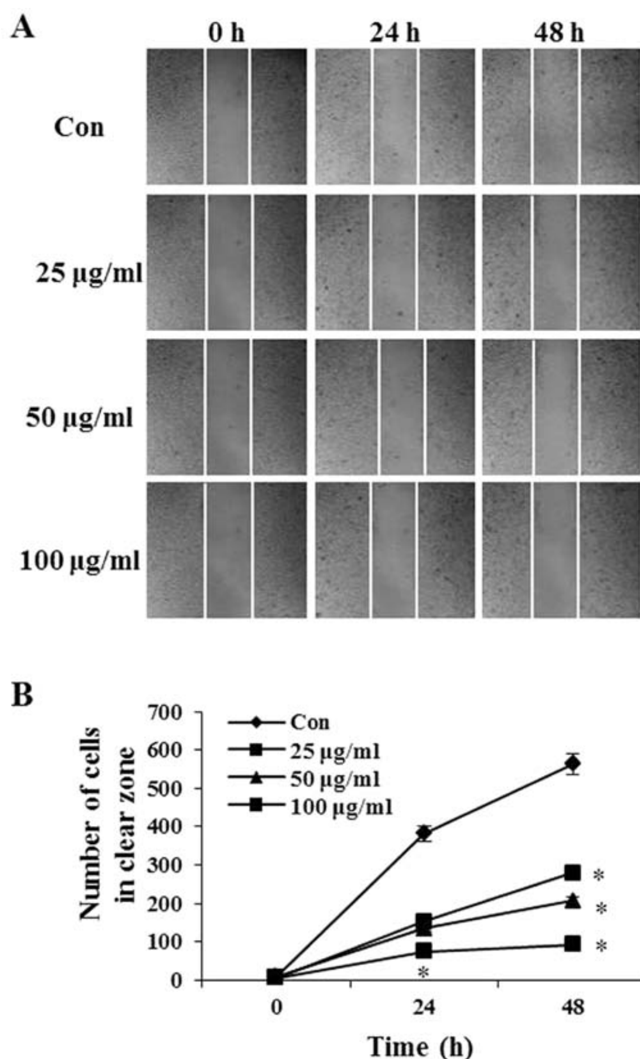

Figure 2. Effects of PIO on the motility of the B16-F10 cells. Cell mobility was determined by the wound healing assay. (A) Monolayers of B16-F10 cells were treated with various concentrations (0, 25, 50 and 100 μg/ml) of PIO for 24 and 48 h. (B) Quantitative assessment of the number of cells in the denuded zone. The data represent the mean ± SE of three independent experiments. *P<0.05 indicates a significant difference from the control.

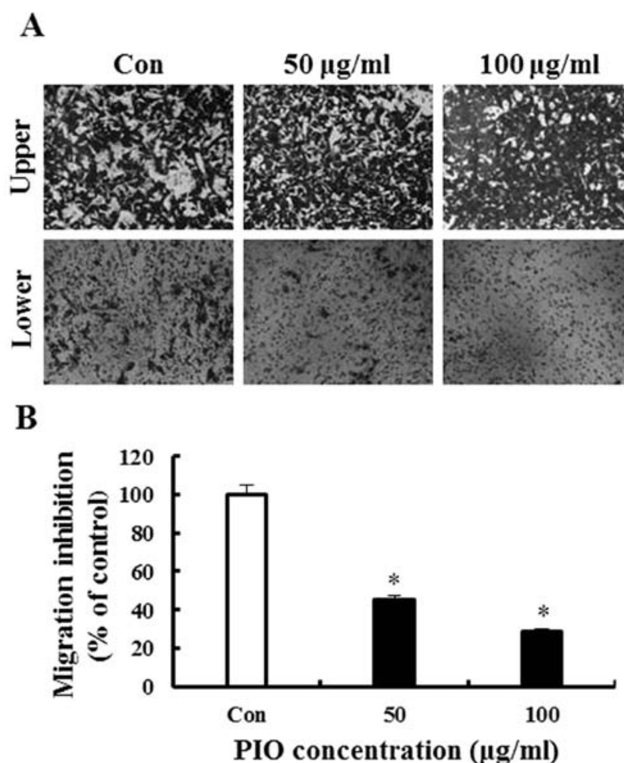

Figure 3. Effect of PIO on the migration of the B16-F10 cells. The B16-F10 cells were treated with various concentrations (0, 50 and 100 μg/ml) of PIO for 24 h. (A) Cell migration was measured for 24 h in a Transwell chamber with polycarbonate filters (pore size, 8 μm) precoated with gelatin. (B) The migratory ability of B16-F10 cells was quantified by counting the number of migrated cells. Cells were captured using an optical microscope (original magnification, x40). The data represent the mean ± SE of three independent experiments. *P<0.05 indicates a significant difference from the control.

Effects of PIO on the motility of B16-F10 cells. The effect of PIO on B16-F10 cell migration was determined using the wound-healing assay in which cells were stimulated to migrate by physical wounding cells. As shown in Fig. 2A, when cells were treated with PBS for 24 and 48 h, an apparent and gradual increase of cells in the denuded zone was observed under light microscopy. B16-F10 cells treated with 25, 50 and 100 μg/ml of PIO displayed a reduced ability to migrate and fill the wounded area compared with untreated cells. The quantitative data in Fig. 2B revealed that PIO significantly inhibited the migration of B16-F10 cells.

Effect of PIO on migration and invasion of B16-F10 cells. To further evaluate the anti-metastatic activity of PIO, we assessed the inhibition of B16-F10 melanoma migration and invasion by PIO using Transwell assay with polycarbonate filters (pore size, 8 μm) precoated with gelatin or Matrigel. The results indicated that B16-F10 cells moved from the upper chamber to the lower chamber in the absence of PIO (control group), suggesting that B16-F10 melanoma cells can migrate across a Transwell insert precoated with gelatin. PIO at 50 and 100 μg/ml significantly inhibited melanoma cell migration by 56 and 72%, respectively (Fig. 3). As shown in Fig. 4, the results of the invasion assay illustrated that untreated B16-F10 cells moved from the upper to the lower chamber, suggesting that B16-F10 cells can invade through Matrigel-coated Transwell cell culture chambers. However, the addition of PIO to the B16-F10 cells resulted in inhibitory effects on cellular invasion in a concentration-dependent manner. Data in Fig. 4B indicate that 50 and 100 μg/ml PIO significantly inhibited invasion by 31 and 61%, respectively. Thus, these results suggested that PIO effectively reduced melanoma cell migration and invasion.

Effect of PIO on the activities and expression of MMPs of B16-F10 cells. ECM degradation is crucial to cellular invasion, indicating the inevitable involvement of matrix-degrading proteinases (27). Therefore, the effect of PIO on MMP activities was investigated by gelatin-zymography under a condition of serum starvation to clarify the contribution of MMPs to the inhibitory effect of PIO on the invasive ability of cells. As shown in Fig. 5A, gelatin-zymography was used to analyze the effect of PIO on MMP-2 and MMP-9 activities in B16-F10 melanoma cells. MMP-2 and MMP-9 activities were markedly reduced by exposure to PIO at concentrations of 25, 50, and

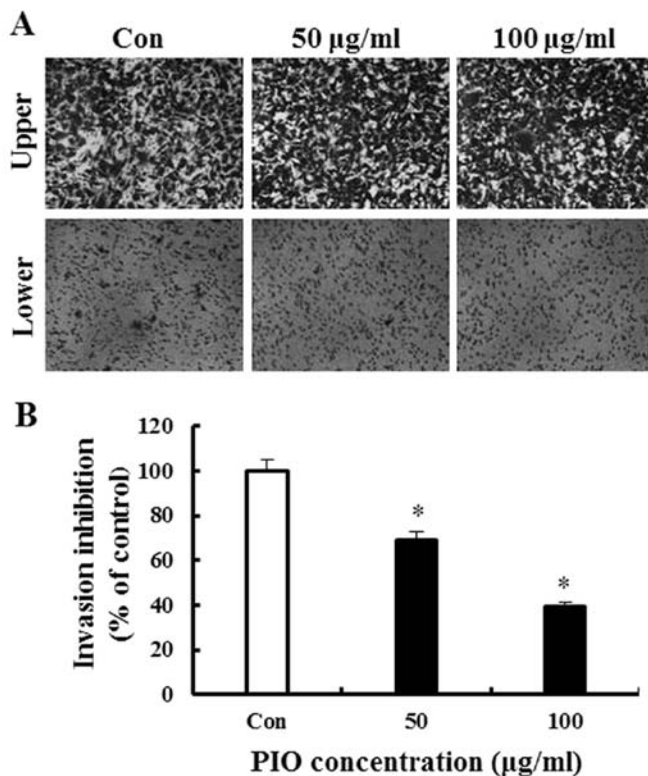

Figure 4. Effect of PIO on the invasiveness of the B16-F10 cells. The B16-F10 cells were treated with various concentrations (0, 50 and 100 μg/ml) of PIO for 24 h. (A) Cell invasion was measured after 24 h in a Transwell chamber with polycarbonate filters (pore size, 8 μm) precoated with Matrigel. (B) The invasiveness of B16-F10 cells was quantified by counting the number of invaded cells. Cells were captured using an optical microscope (original magnification, x40). The data represent the mean ± SE of three independent experiments. *P<0.05 indicates a significant difference from the control.

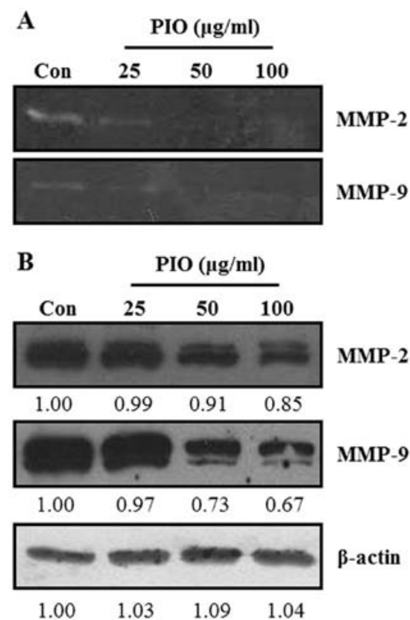

Figure 5. Effect of PIO on the expression and activities of MMP-2 and MMP-9 in the B16-F10 cells. The B16-F10 cells were treated with various concentrations of PIO in serum-free medium for 24 h. (A) The gelatinolytic activities of MMP-2 and MMP-9 were determined by zymography. (B) The expression levels of MMP-2 and MMP-9 were measured by western blot analysis. Equal loading of total proteins in each sample was verified by β-actin expression.

100 μg/ml for 24 h. To further understand the effects of PIO on MMP-2 and MMP-9, western blot analysis was performed. As shown in Fig. 5B, PIO inhibited the expression of MMP-2 and MMP-9 in B16-F10 melanoma cells. Therefore, these results indicated that PIO regulated the expression and activities of MMP-2 and MMP-9.

Effect of PIO on NF-κB nuclear translocation and COX-2 expression levels in B16-F10 cells. Activation of NF-κB in metastatic cancer cells is involved in the expression of MMP-2 and MMP-9 (28). COX-2 also affects the expression of MMP-2 and MMP-9 in highly metastatic cancer cells (13). To investigate whether PIO could regulate the NF-κB signaling pathway in B16-F10 melanoma cells, cells were treated with the indicated concentrations of PIO. The translocation of NF-κB and COX-2 expression levels were determined by western blot analysis. As shown in Fig. 6, the total cytosolic NF-κB protein levels in B16-F10 cells was increased by PIO treatment compared with that in untreated cells. By contrast, the protein levels of NF-κB in the nucleus of B16-F10 cells markedly decreased after PIO treatment compared with the control levels. Moreover, we found that the expression levels of COX-2 in PIO-treated B16-F10 cells were markedly lower than those of the untreated control. These data indicated that PIO inhibited NF-κB activation and COX-2 expression in melanoma cancer cells.

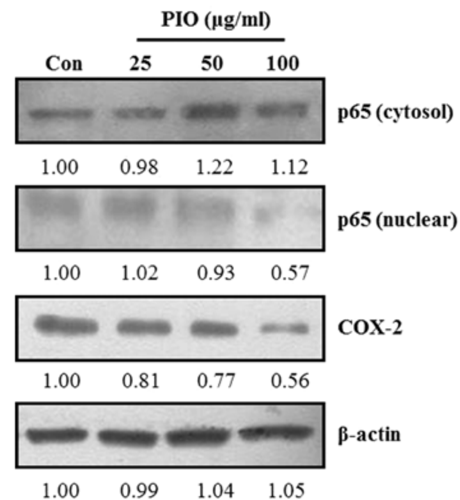

Figure 6. Effect of PIO on the translocation of NF-κB and COX-2 expression in the B16-F10 cells. The B16-F10 cells were treated with various concentrations of PIO for 24 h. The levels of activated NF-κB and COX-2 were determined by western blot analysis using a nuclear extraction kit. Equal loading of total proteins in each sample was verified by β-actin expression.

Effect of PIO on MAPK and Akt signaling pathways in B16-F10 cells. Recent studies reported that MAPK and PI3K/Akt signaling pathways are involved in cancer cell migration and invasion (29). MAPKs and Akt have been demonstrated to be involved in MMP induction in various tumor types (30). To examine whether PIO could regulate MAPK and Akt signaling pathways in B16-F10 melanoma cells, we analyzed the phosphorylation levels of all three MAPKs (ERK, JNK and p38 MAPK) and Akt by western blot analysis using respective anti-phospho MAPK and Akt mAbs in B16-F10 cells after

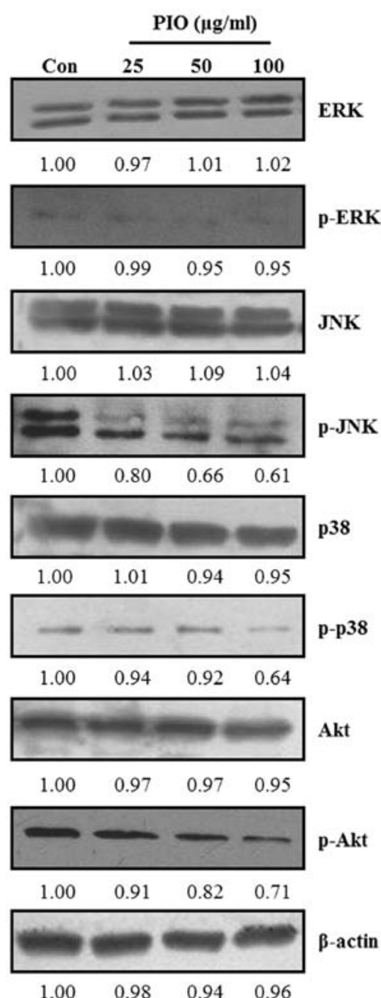

Figure 7. Effect of PIO on Akt and MAPK signaling in the B16-F10 cells. The B16-F10 cells were treated with various concentrations of PIO for 24 h. The levels of activated Akt, ERK, p38 MAPK, and JNK were determined by western blot analysis. Equal loading of total proteins in each sample was verified by β-actin expression.

treatment with PIO (25, 50 and 100 μg/ml) for 24 h. As shown in Fig. 7, PIO did not affect the expression levels of all three MAPKs and Akt, but PIO suppressed the phosphorylation levels of ERK, JNK, p38 MAPK and Akt in comparison with the findings in the untreated control. In particular, the phosphorylation levels of JNK, p38 MAPK and Akt were highly inhibited by the addition of PIO at concentrations of 50 and 100 μg/ml. These data indicated that PIO inhibited the phosphorylation of MAPKs and Akt in melanoma cells.

Discussion

It is well known that tumor metastasis occurs in many steps including vessel formation, cell attachment, adhesion, migration, invasion and cell proliferation (31). These events are regulated by an extremely complex mechanism. Therefore, considerable attention is focused toward developing agents or drugs that can inhibit metastasis; however, anti-metastatic agents are still lacking. Numerous reports have revealed that the inhibition of MMP expression and/or inhibition of the activities of MMP enzymes can prevent cancer metastasis (32,33). MMP-2 (72-kDa gelatinase A) and MMP-9 (92-kDa gelatinase B) are involved in the invasive metastatic potential of tumor cells. These MMPs are also associated with COX-2, the activity and expression of which may modulate the expression and activity of MMPs (34). In the present study, we investigated the effects of PIO on the migration and invasion of the highly metastatic B16-F10 mouse melanoma cell line *in vitro*. Our results from wound healing, migration and invasion assays also demonstrated that PIO inhibited the migration and invasion of B16-F10 cells (Figs. 3 and 4). In addition, the results indicated that PIO can suppress the expression and activities of MMP-2 and MMP-9, which facilitate the degradation of ECM and play important roles in cancer cell migration and invasion (Fig. 5). These findings demonstrated that the anti-metastatic effects of PIO were associated with the inhibition of enzymatic degradation processes in metastatic B16-F10 cells and suggest that PIO may be efficacious at preventing the metastasis of cancer cells. We found that PIO also regulated potential signaling pathways related to the migration and invasion of highly metastatic cancer cells (Fig. 7). PIO inhibited the phosphorylation of MAPKs. The MAPK signaling pathway was found to promote tumor invasion and metastasis in B16-F10 cells. We also found that PIO inhibited the phosphorylation of Akt in B16-F10 cells, indicating that PIO could inhibit the Akt signaling pathway. It was reported that PI3K activation stimulated the downstream target AKT, which is associated with cell invasion. It is well documented that the PI3K-Akt pathway plays important roles in the invasive properties of cancer cells (35). Previous reports demonstrated that the MMP-2 and MMP-9 promoter has several transcription factor-binding motifs including those for NF-κB and AP-1. Multiple pathways leading to the activation of NF-κB and AP-1 binding factors in tumor cells may contribute to MMP-2 and MMP-9 transcription and enhance invasiveness (36). In the present study, we found that PIO regulated NF-κB translocation from the cytosol to the nucleus and the expression and activities of MMP-2 and MMP-9 in B16-F10 cells *in vitro*. Thus, PIO may suppress the mRNA expression of MMP-2 and MMP-9 via RNA transcription factors. These findings indicate that PIO strongly inhibits the metastasis of B16-F10 cells by inhibiting NF-κB translocation. Then, PIO reduces the transcription and translation of MMP-2 and MMP-9, thereby decreasing the activities of MMP-2 and MMP-9. These results suggest that PIO inhibited the expression and activities of MMP-2 and MMP-9 by inhibiting the NF-κB signaling pathway. Evidence indicates that COX-2 plays an important role in the carcinogenesis and progression of cancer, and the induction of MMP-2 and MMP-9 expression may be a mechanism by which COX-2 promotes the development and metastasis of cancer (37). The present study demonstrated that PIO markedly reduced *in vitro* COX-2, MMP-2 and MMP-9 expression in B16-F10 cells. Furthermore, migration and invasion data confirmed that PIO significantly inhibited the migration and invasion of B16-F10 cells. In conclusion, MMPs play an important role in tumor metastasis. Therefore, it is well established that MMP gene expression and enzymatic activity are early targets for preventing cancer metastasis. The present study suggested that PIO inhibits the migration and invasion of highly metastatic B16-F10 cells, which may mainly result from the PIO-mediated inhibition of the expression and activities of MMP-2 and MMP-9 via the suppression of Akt, MAPKs NF-κB and COX-2 signaling pathways.

Acknowledgements

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