

Reduction of matrix metalloproteinase-9 expression by culture filtrate of *Paecilomyces farinosus* J3

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Abstract. The aim of the present study was to investigate the anti-tumor effects of a culture filtrate of *Paecilomyces farinosus* J3. Various anti-tumor assays using B16 melanoma cells were carried out. *Paecilomyces farinosus* J3 significantly decreased the wound healing capability, invasiveness and angiogenic activity, which was confirmed by wound healing, human umbilical vein endothelial cell and invasion assays. *Paecilomyces farinosus* J3 strongly inhibited cell migration, tube formation and the angiogenic process in a concentration-dependent manner. Zymographic analysis also indicated a reduced expression of matrix metalloproteinase-9 (MMP-9), a 92-kDa gelatinase. Taken together, the results indicate that the anti-tumor activities of *Paecilomyces farinosus* J3 originate from the reduction of MMP-9 expression in B16F10 cells.

Introduction

Cancer is a progressive disease that affects many types of tissues, as well as organs, in the human body (1). Cancer progression and metastasis is a complicated process. Cells move through the blood and invade neighboring tissue by degrading the extracellular matrix (ECM) (2). In these multi-processes, angiogenesis plays a pivotal role in the formation of new blood vessels in the newly colonized areas, and thus in the formation of additional metastatic lesions (3,4). The ECM includes a wide variety of proteins, which can be degraded by

many matrix metalloproteinases (MMPs). MMPs are a family of highly conserved zinc-dependent peptidases capable of degrading the ECM (3).

Anti-tumor agents have been shown to inhibit MMP-9 activity in the ECM via the binding of the NF- κ B transcription factor with the MMP-9 promoter (5). This suggests that anti-tumor agents play a role in anti-invasion and/or anti-angiogenesis, as well as in caspase-3 inhibition (6). Cell motility, and in particular cell migration, affect tumor progression and metastasis (7). The signaling pathways responsible for regulating cell motility involve various nuclear transcription factors, as well as morphological changes in the cytoskeleton that modify interactions among membrane- and ECM-bound proteins (e.g., cdc, Rho and Rac family proteins), which mediate cellular and membrane morphology by controlling the polymerization-depolymerization process of the actin cytoskeleton (8).

Cordyceps and *Paecilomyces* sp. are popular entomopathogenic fungi, which have been broadly called DongChungHaCho (winter worm summer grass) in Korea (8-11). *Paecilomyces* sp. has been investigated for industrial purposes in order to be cultivated on a large scale. However, the precise biological mechanisms of functionality and bioavailability require investigation.

In this study, we show that *Paecilomyces farinosus* J3, which was collected and isolated from Mansusan, Korea, decreases cell migration, invasion and tube formation, suggesting that this activity may have originated from the pathway of MMP-9 inhibition. Our findings, in combination with previous studies, strongly suggest that *Paecilomyces farinosus* J3 should be considered a good candidate for development into an anti-tumor therapeutic agent, as well as in anti-tumor preventive medicine, provided that MMP-9 is the major molecular target molecule for angiogenesis and tumor progression.

Materials and methods

Culture and separation of *Paecilomyces* sp. The strain *Paecilomyces farinosus* J3 was collected from Mansusan

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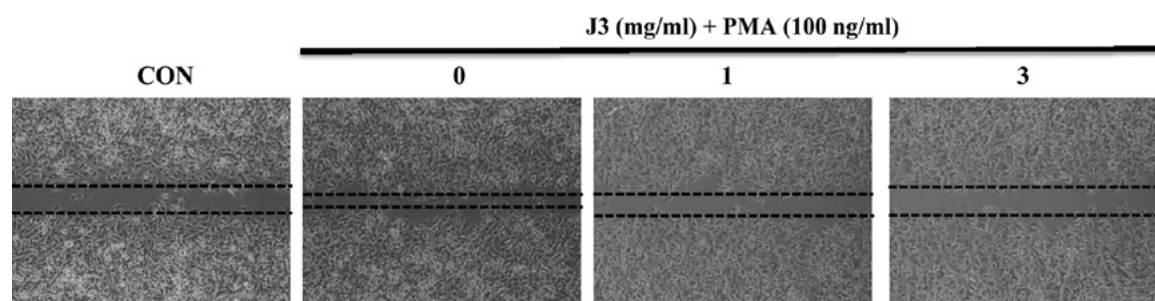


Figure 1. *Paecilomyces farinosus* J3 inhibits cell migration *in vitro*. (A) Confluent monolayers of B16 cells were pre-treated with or without the *Paecilomyces farinosus* J3 culture filtrate. The monolayers were then wounded and stimulated with 100 ng PMA or left untreated (controls). The plates were photographed at 0 or 24 h post-wounding. Bar, 200 μ m. (B) Quantification of the wound healing. Cell migration was quantified by counting the wound width 24 h after the plates were treated with or without PMA. Values are the means \pm SD from five cultures each in duplicate experiments. CON, control. *Significant difference from the control, $P < 0.05$. **Significant difference from PMA treatment alone, $P < 0.05$.

and deposited in the Collection Center of the Department of Agricultural Biology, National Advanced Institute of Science and Technology, RDA, Suwon, Korea. The strain was cultured and re-isolated in PDA medium (24 g potato dextrose, 15 g agar and 1 l distilled water) at 25°C for 2 weeks. After sufficient maturation of the mycelial growth, the culture supernatants were centrifuged and divided into mycelial extract and culture filtrate. The culture filtrate was then freeze-dried under a vacuum evaporator. The freeze-dried powder of culture filtrate was dissolved in distilled water prior to use (12).

Cell culture. The mouse melanoma cell line B16F10 (B16; Catalog #CRL-6323) and human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The B16F10 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, while the HUVECs were cultured in EGM-2 medium (BD Biosciences, Lake Placid, NJ, USA). The B16F10 cells were maintained in RPMI-1640 medium and subcultured by trypsinization at 3- to 4-day intervals. The HUVECs were subcultured every 3-4 days and were used for experiments at passages 3-10 (13).

Wound healing assay. The strips of sterilized thin film tape (2 mm x 2 cm; 3M, Seoul, Korea) were attached to the bottom of each well of 6-well plates (Greiner, Frickenhausen, Germany), and the B16 cells were split into plates at a concentration of 1×10^7 cells/well, and allowed to grow for 6 h at 37°C in a 5% CO₂ atmosphere. The tape strips were then detached, creating linear wounds. The plates were photographed and incubated as described above with medium containing various concentrations of J3 (0-100 mg/ml). The plates were photographed at 24 h and the precise wound width was calculated by use of a microruler (<http://www.eeob.iastate.edu/faculty/DrewsC/htdocs/microruler-links.htm>) as described previously (14).

Invasion assay. Transwell plates (pore size 8 μ m; Costar, NY, USA) were loaded with 100 μ l Matrigel (BD Biosciences), allowed to solidify at 37°C for 2 h and then coated with 10 μ l of fibronectin (200 μ g/ml). The plates were loaded with B16 cells suspended in 10% FBS (1×10^5 cells/well), the samples were exposed to J3 (0-100 mg/ml) and the plates were incu-

bated at 37°C in 5% CO₂ for 24 h. The migrated cells were fixed with methanol, stained with hematoxylin and counted under a microscope (15).

Tube formation assay. HUVECs (2×10^4 cells/well) were added to the Matrigel-coated 24-well plates in 0.5 ml of EGM-2 medium with various concentrations of J3 (0-100 mg/ml) and incubated for 24 h. The cells were then visualized under a microscope and tube formation was scored by counting the number of tubes formed (14,15).

Gelatin zymography. Gelatin zymography was performed as described previously (16). Proteins with gelatinolytic activity were identified by electrophoresis in the presence of SDS on 8% polyacrylamide gels containing 0.1% (w/v) gelatin. In brief, cell culture medium was obtained from cultured B16F10 cells in DMEM and PMA (100 ng), with or without different concentrations of J3 for 24 h in 6-well culture dishes. The cultured medium was mixed with an SDS-PAGE sample buffer in the absence of β -mercaptoethanol and DTT. Five microliters of sample were loaded onto a gel and electrophoresed. After electrophoresis, the gel was renatured twice for 30 min at room temperature in 2.5% (v/v) Triton X-100, and subsequently transferred onto a zymogram-developing buffer containing 50 mM Tris-HCl, 5 mM CaCl₂, 0.2 M NaCl and 0.02% Brij 35 at 37°C overnight. After staining with Coomassie Blue R-250 (0.25%) for 30 min, the gel was then destained in a destaining solution [methanol:acetic acid:water (50:10:40)]. A clear band was detected and quantified using the BioRad GelDoc system.

Statistical analysis. Data are expressed as the means \pm standard deviation (SD). Statistical significance was determined by the Student-Newman-Keuls method for independent means, using the Sigma Plot program (17). The critical level of significance was set at $P < 0.05$.

Results

In a previous study, we developed various cultivation methods and cultivated various insect pathogenic fungi, including *Paecilomyces* sp. (DongChungHaCho in Korean). The morphology of the selected strain has a classical feature of mycelial growth that is common in entomopathogenic fungi

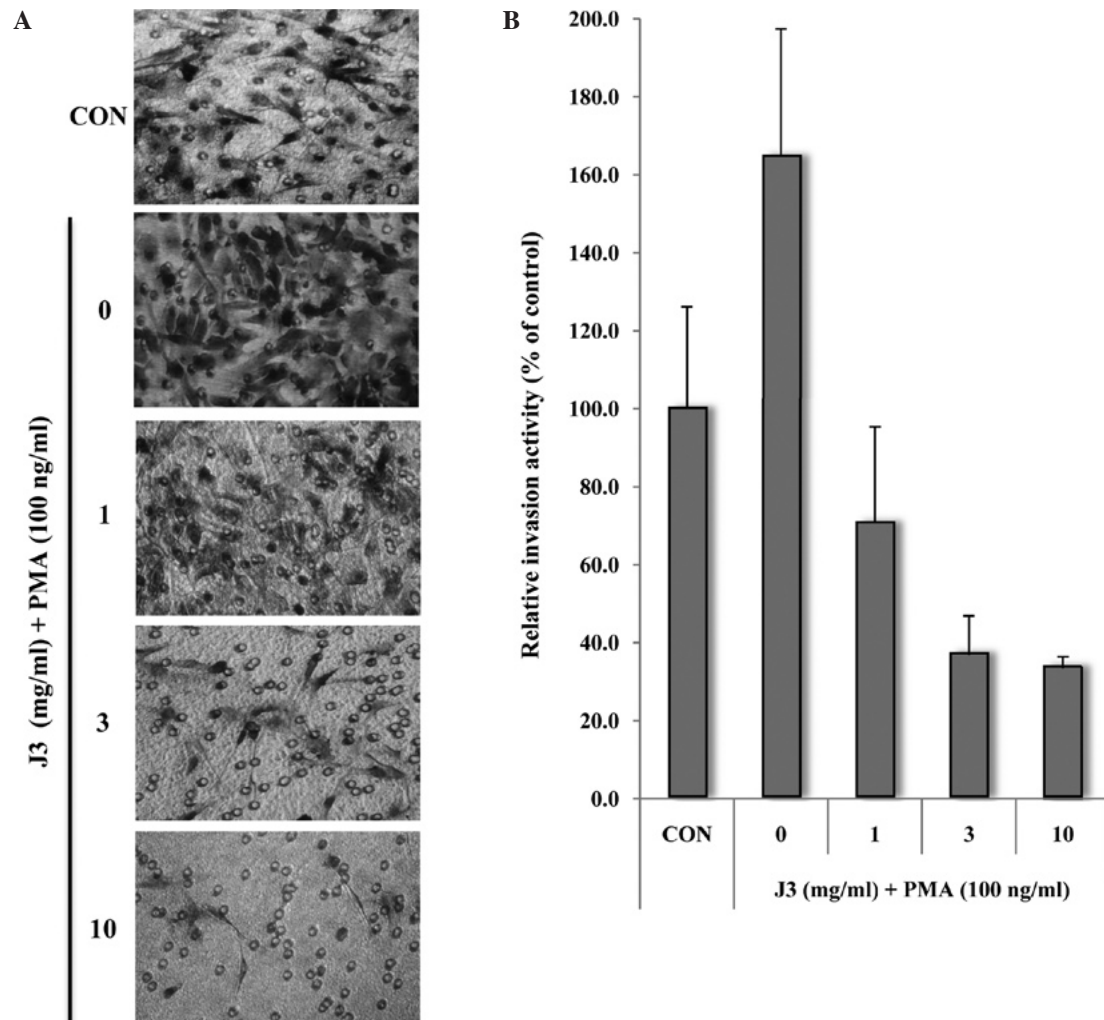


Figure 2. *Paecilomyces farinosus* J3 inhibits cell invasion *in vitro*. Cell invasion was assayed in a Matrigel-coated Transwell chamber. (A) Invasion was compared among B16 cells exposed to no treatment (control; a), 100 µg/ml of *Paecilomyces farinosus* J3 (b), 75 nM PMA (c) and both *Paecilomyces farinosus* J3 and PMA (d). Representative fields of migrated cells were photographed. Bar, 200 µm. (B) Quantification of the invasion activity. Cell migration was quantified 24 h after the cells were exposed to no treatment (control; CON), *Paecilomyces farinosus* J3, PMA, or both *Paecilomyces farinosus* J3 and PMA. Migrated cells were counted from five randomly selected microscopic fields and the results are given as the average per field ± SD of three independent experiments. *Significant difference from the control, $P < 0.05$. **Significant difference from PMA treatment alone, $P < 0.05$.

(data not shown). In this study, we optimized the maintenance and culture medium of *Paecilomyces farinosus* J3 (data not shown), and investigated the biological activities of the culture filtrate of that strain. Few strains have been studied for their anti-tumor activities and their efficacy as culture filtrates.

We first examined the tumor cell motility of J3-treated B16 cells. We used a wound healing assay to assess the *in vitro* effect of J3 on tumor cell migration, which is an important phenomenon of cancer cell motility, particularly in relation to metastatic cancer. The wound healing assay showed that the monolayers treated with 10 mg/ml of J3 displayed a clear wound width, while the untreated control monolayers exhibited complete wound healing within 24 h, indicating that at J3 partially inhibits cell migration *in vitro* in a dose-dependent manner (data not shown). Notably, the PMA-treated monolayers also showed a cell growth inhibitory pattern (Fig. 1, 2nd to 4th lower images) as did the control, suggesting that J3 inhibited cell migration activity with or without a cell proliferation inducer. Similarly, the invasion assay revealed that J3 treatment (10 mg/ml) inhibited invasive activity by >40%

vs. the control group (Fig. 2A). These results indicate that J3 dose-dependently alleviates wound healing and inhibits invasion *in vitro* in a concentration-dependent manner (Fig. 2B).

We then investigated the effect of J3 on angiogenesis, which is a key feature of metastasis. HUVECs were treated with or without various concentrations of J3, and tube formation was measured in terms of tube size and number. The data demonstrated that J3 inhibits HUVEC tube formation *in vitro* in a dose-dependent manner (Fig. 3; 0.1-3 mg/ml). Notably, the cell tubular numbers were dramatically decreased by various doses of J3 (Fig. 3B, compare columns 2 and 5). Collectively, these findings indicate that J3 inhibits cell migration, cell invasiveness and tube formation *in vitro*, suggesting that this extract can function as an anti-tumor component *in vitro* by decreasing metastatic and/or angiogenic potential.

Zymographic analysis revealed that the treatment of cells with 0.1-10 mg/ml of J3 inhibited gelatinase (MMP-9; 92 kDa) by ~45% (Fig. 4A), suggesting that J3 has a strong potential to hamper the secretion of the active form of MMP-9 rather than the activity level *per se*. The relative MMP-9 activity reached

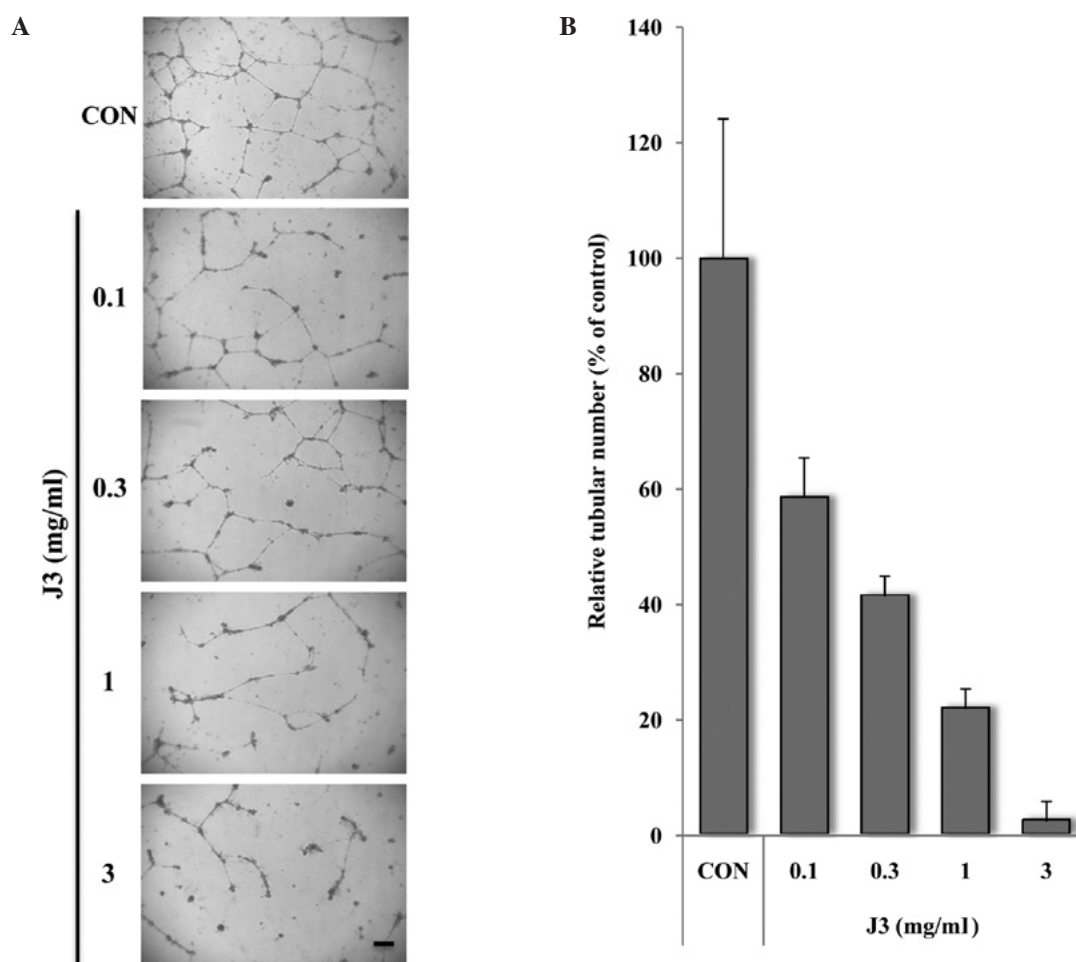


Figure 3. *Paecilomyces farinosus* J3 alleviates HUVEC tube formation on Matrigel. (A) HUVECs were plated at 2×10^4 cells/well in a Matrigel-coated 24-well plate, and then exposed to 0, 0.1, 0.3, 1 or 3 mg/ml of *Paecilomyces farinosus* J3 culture filtrate. After 24 h, the culture medium was removed and the cells were fixed with 10% formalin. The cell morphology infiltrated into the Matrigel (A), and the relative tubular numbers (B) of the formed tubes were calculated. Bar, 10 μ m. The results are shown as the average per field \pm SD of three independent experiments. CON, control. *Significant difference from the control (*Paecilomyces farinosus* J3, 0 mg/ml), $P < 0.05$.

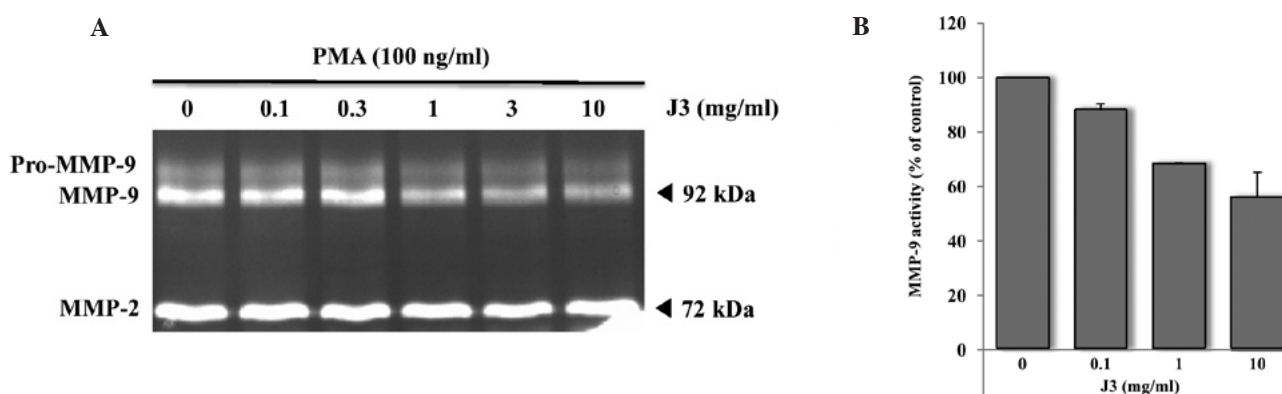


Figure 4. *Paecilomyces farinosus* J3 inhibits MMP-9 expression. (A) Proteins with gelatinolytic activity were identified by electrophoresis in the presence of SDS on 10% polyacrylamide gels containing 0.1% (w/v) gelatin. In brief, cell culture medium was obtained from cultured B16F1 cells in DMEM and PMA (75 nM), with or without different concentrations of J3 for 24 h in 6-well culture dishes. The cultured medium was mixed with SDS-PAGE sample buffer in the absence of β -mercaptoethanol and DTT. Five microliters of sample were loaded onto a gel.

a level of 55% of the control at a concentration of 10 mg/ml (Fig. 4B). MMP-2 activity did not change by J3 treatment with or without PMA (Fig. 4B; data not shown). Additionally, the expression of other MMPs, as well as other tumor-related

molecules (uPAR, Timp-1, Timp-2, Paxillin, Src and ARF-2), did not change after treatment with J3 (data not shown). However, the precise up- and down-stream signaling pathway involved needs to be investigated.

Discussion

Traditional medicine utilizing plant and/or herbal extracts is believed to inhibit cancer cell growth and control the homeostasis of malignant cells in tissues or organs (1,2). For many years, herbal extracts have been used as functional food materials in Oriental medicine, in order to maintain homeostasis in the body and to mitigate the symptoms of degenerative diseases (18,19). Moreover, the tyrosinase-inhibitory activity of *Cordyceps sp.* extracts leads to the suppression of melanin production (20); these extracts therefore have a potential use in cosmetics. Additionally, the anti-depressant, anti-oxidative, anti-hepatic, anti-diabetic and anti-aging activities of various extracts of *Cordyceps sp.* lead to a wide range of bioactivities *in vitro*, as well as *in vivo* (21-25). *Paecilomyces sp.* is also a type of entomogeneous fungus. The bioavailability and functionality of *Paecilomyces sp.* are more useful and potent than those of *Cordyceps sp.*, in that the strain has beneficial effects on artificial cultivation and usefulness in host infections (26-29). This study was undertaken to investigate the anti-tumor effects of the culture filtrate of *Paecilomyces farinosus* J3. The results of a wound healing assay, invasion assay, HUVEC assay and gelatin zymography indicated that J3 inhibited B16 melanoma cell growth, thus demonstrating its anti-tumor potential. We therefore sought to examine the mechanisms involved in this cell growth inhibition by J3.

First, we examined the effect of the culture filtrate of J3 on cell migration using a wound healing assay in B16F10 melanoma cells. This cell line is a good model for the assessment of cell morphology, motility and proliferation (13-15). B16 cells migrated into the damaged wound, suggesting that migration is associated with various events in the cytoskeletal structure, such as actin polymerization and changes in the cell motility-related framework (30). J3 exerted a dose- and time-dependent inhibition of wound healing in B16 cells. This phenomenon strongly suggests that certain components in J3 are able to restrict cell motility, by means of cytoskeletal change and/or morphological features.

We investigated whether J3 can influence HUVEC differentiation into capillary-like tube formation. As metastatic potential is characterized by endothelial cell differentiation, *in vitro* angiogenic assays were performed to assess this potential with HUVECs by incubating the cells for 24 h with J3 on Matrigel. As capillary-like structures were observed in the HUVECs in the control, we attempted to confirm this by treatment with J3. As shown in Fig. 3A, in cell morphology the J3-treated HUVECs was markedly decreased in a dose-dependent manner, suggesting that the tube formation was affected by J3 in a concentration- and time-dependent manner (Fig. 3B and data not shown).

Invasion assay are well-recognized *in vitro* angiogenic method for assessing invasion in B16F10 melanoma cells. Fig. 2 elucidates the J3 dose-dependent inhibition of cell invasion in B16F10 melanoma cells on Matrigel in a transwell assay. PMA-activated B16F10 cells underwent a 1.6-fold growth increase compared to the PMA non-treated control cells, whereas J3-treated cells (10 mg/ml) were inhibited via the process of the breakdown of the matrix at a level of 35% compared to the control, and a level of 21% compared to the

PMA-treated cells, although the activity was milder than that of 50 μ M dykellic acid (5,6; data not shown).

There are many types of matrix-degrading proteases in numerous types of cells (3). Of these, gelatinase is now well-known for its role in angiogenesis development, as the enzyme is activated during its passage through the blood vessels when tumor cells are under hypoxic conditions (4). We therefore investigated the effects of J3 on MMPs. RT-PCR analysis of MMP-1 to -28 revealed that only the expression of MMP-9 was reduced (data not shown). In Fig. 4A, by treatment with J3, the MMP-9 level (92 kDa) was decreased in a dose-dependent manner, whereas the MMP-2 level was not. B16F10 melanoma cells did not secrete their pro-MMP-9 form into the medium, and this was not detected in the zymogram (Fig. 4A, above each 92 kDa band). We previously showed that Oriental herbal medicines exhibit various anti-tumor activities. As chemicals from herbal extracts, such as dykellic acid (5,6), methylselenol (31), gall extract of *Wisteria floribunda* (13), methylene chloride fraction of *Geum japonicum* Thunberg (14) and aqueous extract of *Gastrodia elata* Blume (15) are potentially toxic to tissues or organs, we sought to identify natural substances or food-borne biomaterial(s). Among the candidates is J3, since this fungus has no toxicity, as shown by animal experiments and single dose acute/chronic acute toxicity tests (data not shown).

In conclusion, we herein demonstrate that *Paecilomyces farinosus* J3 dose-dependently inhibits B16 cell migration and motility, and inhibits HUVEC tube formation *in vitro*, resulting in decreased levels of MMP-9. These findings, in combination with those of previous studies indicating that J3 inhibits a PMA-induced increase in MMP-9 expression, suggest that J3 may be a promising candidate for future development as an anti-tumor agent.

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