Extract of the mycelium of *T. matsutake* inhibits elastase activity and TPA-induced MMP-1 expression in human fibroblasts

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Received June 2, 2014; Accepted September 24, 2014

DOI: 10.3892/ijmm.2014.1969

Abstract. Skin aging is induced through complex biological processes in human skin caused by proteolysis of collagen and elastin, two structural proteins of the dermal extracellular matrix (ECM). Collagen and elastin degradation can induce the expression of matrix metalloproteinases (MMPs), as well as that of a family of zinc-dependent endopeptidases that play critical roles in skin aging. Moreover, elastase is a metalloproteinase which acts on the degradation of elastin in skin aging, and is also involved in the inhibition or the repair of wrinkle formation. Extract of the mycelium of *Tricholoma matsutake* (*T. matsutake*), or pine mushroom, is widely distributed in Asian countries. The extract is from the natural biomaterial of the mushroom which is rich in polysaccharides, including β-glucan. This extract has shown potent bioactive antioxidant, immunomodulatory and antitumoral properties. In the present study, we investigated whether the extract of the mycelium of *T. matsutake* has effects on elastase activity, as well as on the expression of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) and MMP-1 under basal conditions. Our results revealed that the extract of the mycelium of *T. matsutake* significantly decreased elastase activity in a dose-dependent manner and reduced the levels of MMP-1 and MMP-3. On the other hand, the expression of TIMP-1 and tropoelastin was increased in fibroblasts treated with the extract of the mycelium of *T. matsutake*. However, collagent expression was not affected. In addition, our results demonstrated that the extract of the mycelium of *T. matsutake* inhibited the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced MMP-1 expression and suppressed TPA-induced p38 activity. Therefore, the inhibitory effects of the extract of the mycelium of *T. matsutake* on MMP-1 induction are mediated by the inhibition of p38 in human fibroblasts. Our data suggest that the extract of the mycelium of *T. matsutake* may prove to be an effective biomaterial for anti-wrinkle treatment, as it can obstruct the degradation of the dermal ECM.

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Key words: human fibroblast, aging, 12-*O*-tetradecanoylphorbol-13-acetate, matrix metalloproteinase-1, tissue inhibitor of matrix metalloproteinases-1, elastase, tropoelastin

Introduction

Skin aging occurs through two independent, complex biological processes in the human skin, specifically chronological or intrinsic aging and photo-aging (extrinsic aging) (1). The main events involved in intrinsic aging are enhanced matrix metalloproteinase (MMP)-1 activity and decreased collagen production (2). The symptoms of intrinsic aging are the wrinkling and sagging of the skin, ultimately resulting in the loss of flexibility and elasticity (3,4). The other type of aging is caused by environmental factors, such as ultraviolet (UV) irradiation, gravity and smoking (5). This also plays an important role in wrinkle formation as the synthesis of MMPs accumulates through UV (6).

Human skin is composed of the epidermis and dermis; the dermis is made up of not only collagen, but also elastin. Dermal fibroblasts are produced by the extracellular matrix (ECM), which generates collagen and elastin fiber in tissue and represents the extracellular part of multicellular structure (7). Collagen and elastin from the dermal ECM and have been shown to play a role in regeneration and remodeling (8). Collagen is the most abundant structural protein. Its degradation can be induced by MMPs, which remodel the ECM; several MMPs are known to regulate collagen proteolysis (9). The MMPs are produced by several cell types, including epithelial cells, fibroblasts, neutrophills and mast cells, as well as a family of zinc-dependent endopeptidases that play critical roles in inflammation, tumor invasion and skin aging (10). MMPs have been classified into diverse groups in human skin *in vivo* and *in vitro*, including MMP-1 (collagenases-1), MMP-3 (stromelysin 1 and progelatinase) and MMP-9 (gelatinase B) (11,12). They are inhibited by chelating agents and tissue inhibitors of matrix metalloproteinases (TIMPs), which are known as endogenous inhibitors of the ECM, particularly in the case TIMP-1 and TIMP-2 (13). Elastic fibers consist of two morphologically and chemically distinct components, elastin and microfibrils (1). Elastin is major component of elastic fibers, and greatly contributes to the elastic recoil properties of skin; it is composed of granular amorphous elastin

structures, and is also involved in the inhibition or repair of wrinkle formation. However, elastase is a degrading enzyme present in the dermis, and plays a role in the degradation of the ECM, which contains elastin (14,15). For that reason, it has been reported that it affects the degradation of collagen and elastin in dermal fibroblasts, and that its proteolysis contributes to decreased winkle formation.

The mycelium of *Tricholoma matsutake* (*T. matsutake*), a high-class edible mushroom which has bioactive components, grows throughout late autumn in pine forests, and is widely distributed in Asian countries. It has been reported that polysaccharide extracts from the mycelium of *T. matsutake* exhibit regulatory activity, with immunomodulatory effects in macrophages (16,17). The mycelium of *T. matsutake* has also been reported to have excellent biological activities; the extract from the natural biomaterial of this mushroom is rich in polysaccharides, such as β-glucan. Moreover, β-glucan is presently available as an ingredient in cosmetic formulations and is found in extracts from mushroom, yeasts, various fungi, cereals and seaweed (18). The extract of the mycelium of *T. matsutake* also has strongly bioactive properties, exerting antioxidant, cholesterol-lowering, heart disease prevention, and antitumoral, anti-aging, anti-wrinkle and anti-acne effects (16,19,20). The most promising treatments in skin aging include herbal extracts, several vitamins and antioxidant food supplements, which have been widely accepted to enhance collagen and elastin production in the ECM and reduce the levels of dermal enzymes, as MMPs and elastase, thereby restoring skin elasticity and delaying the process of wrinkle formation. Therefore, natural anti-wrinkle or anti-aging formulations can be developed to reverse the effects of skin aging. In this study, we demonstrate that treatment with extract of the mycelium of *T. matsutake* inhibits 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced MMP-1 gene expression. We also investigated the anti-aging properties of the mycelium of *T. matsutake* and whether they are mediated through the expression of TIMP-1 and tropoelastin.

Materials and methods

Materials. Antibodies against MMP-1 (ab53142), MMP-3 (ab52915), TIMP-1 (ab61224), tropoelastin (ab21600), and collagen I (ab292) were purchased from Abcam, Inc. (Cambridge, MA, USA). The antibody to GAPDH (sc-20357) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The antibody to tropoelastin purchased from Elastin Products Company Inc. (Owensville, MO, USA). Reagents including thiazolyl blue tetrazolium bromide (MTT), TPA, Tris-HCl, sodium dodecyl sulfate (SDS), β-mercaptoethanol, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Na_3VO_4), sodium fluoride (NaF) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). MMP inhibitor I (#444250) and p38 inhibitor (SB203580) were obtained from Calbiochem (San Diego, CA, USA). The synthetic substrate for N-succinyl-tri-alanyl-p-nitroaniline (STANA) was purchased from the Peptide Institute Inc. (Osaka, Japan). Phosphoramidon was obtained from Boeringer Mannheim (Mannheim, Germany).

Preparation of extract of the mycelium of T. matsutake. The extract of the mycelium of *T. matsutake* was purchased from NovaCell Technology Inc. (Seoul, Korea). The dried plant material (20 g) was cut into small sections and extracted using a shaking incubator at 37˚C for 24 h. The extract was then centrifuged at 6,000 x g for 10 min and processed through filter paper and freeze-dried to yield a powdered extract.

Cell isolation and culture of fibroblasts. Human fibroblasts were isolated from human donors aged 30 to 40 years, obtained during plastic surgical procedures. Skin specimens were processed according to the methods described in the study by Rheinwald and Green (21), and the procedure was modified by the use of thermolysin (Sigma-Aldrich Co.). Enzyme activity was inactivated using Dulbecco's modified Eagle's medium (DMEM; WelGENE Inc., Daegu, Korea) with 10% fetal bovine serum (FBS; HyClone Inc., Logan, UT, USA) and centrifuged at 1,200 x g for 10 min to obtain a pellet. The cell pellet was resuspended in medium and incubated at room temperature, and the cellular remains were filtered through a 100 μm nylon mesh. The filtered cells were collected by centrifugation at 1,200 x g for 10 min. The resuspended cells were maintained in high-glucose (4.5 g/l) DMEM. Normal human fibroblasts were cultured in DMEM, supplemented with 10% FBS, 1% penicillin (10,000 U/ml) and 1% streptomycin (10,000 g/ml). The cultures were maintained at 37˚C in a humidified 5% CO_2 incubator.

MTT assay for cell proliferation. Cell proliferation was determined using the MTT reduction assay. To measure cell proliferation, human fibroblasts ($1x10^4$ cells/well) seeded in 24-well plates for 24 h were incubated in DMEM containing extract of the mycelium of *T. matsutake* for 72 h at 37˚C in 5% CO_2. Following serum starvation for 24 h, the cells were incubated with the test substances for the indicated periods of time at 37˚C in 5% CO_2. Subsequently, 100 μl of MTT at 5 mg/ml were added to each well, and incubation was continued for 4 h. The supernatants were removed and formazan crystals resulting from mitochondrial enzymatic activity on MTT substrate were solubilized with dimethylsulfoxide (DMSO; Sigma-Aldrich Co.). Absorbance was measured at 540 nm using an ELISA reader (VERSAMax; Molecular Devices, Sunnyvale, CA, USA).

Measurement of elastase activity. Elastase activity was evaluated using isolated elastase from human fibroblasts at passage 4 and measured using the substrate, STANA (Peptide Institute Inc.), as previously described in the study by Tsuji *et al* (22). Fibroblast-derived elastase activity was measured following treatment of the fibroblasts with the extract of the mycelium of *T. matsutake* at various concentrations (0.1-100 μg/ml) in DMSO. The cells were dissolved in 0.1 M phosphate buffer (pH 6.8) and the supernatant was then used as the enzyme source. To measure the elastase activity, 200 μl of enzyme solution were distributed into each well of a 96-well plate and then pre-incubated for 15 min at 37˚C with or without inhibitors. Following the addition of 2 μl of 62.5 mM STANA, the plates were further incubated for 1 h at 37˚C. The release of p-nitroaniline was measured by the absorbance at 405 nm, and the enzymatic activity was expressed as units per milligram of protein. Each assay was carried out in triplicate.

Western blot analysis. Human fibroblasts were prepared in cell lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA and protease inhibitors (Roche Diagnostics Inc., Indianapolis, IN, USA)]. SDS-polyacrylamide gel electrophoresis was performed using 10 μg of protein per lane. The gels were blotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) that were then saturated with 5% dried milk in Tris-buffered saline with 0.5% Tween-20 (Sigma-Aldrich Co.) The blots were incubated with the appropriate primary antibodies against collagen I, MMP-1, MMP-3, TIMP-1 and GAPDH overnight at 4˚C and then further incubated with horseradish peroxidase-conjugated secondary antibodies after washing. Bound antibodies were detected using a Super Signal West Pico chemiluminescence substrate (Pierce Biotechnology Inc., Rockford, IL, USA).

Immunofluorescence staining. The cells ($5x10^4$) were seeded onto glass coverslips that were pre-coated with poly-L-lysine (0.01%; Sigma-Aldrich Co.) and incubated for 24 h. The cells used for immunefluorescence staining were fixed with 4% paraformaldehyde for 30 min at room temperature. After 30 min, the cells were washed with PBS, and then treated for 10 min in 0.01% Triton X-100 (Sigma-Aldrich Co.) The cells were blocked for 30 min with 5% bovine serum albumin (BSA; Sigma-Aldrich Co.) and incubated overnight at 4˚C with an antibody against collagen I at 1:100 in 5% BSA. After washing, the cells were further incubated with FITC-labeled secondary antibody (Santa Cruz Biotechnology, Inc.) for 2 h and then stained with 4,6-diamino-2-phenylidole (DAPI; Pierce Biotechnology Inc.) at 1 μg/ml for 10 min. Cell morphology was observed under a a DP70 fluorescence microscope and DP Controller software (Olympus Optical Co., Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from the cultured human fibroblasts using an RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA). Subsequently, 1 μg of RNA was reverse transcribed using AccuPower RT-PCR Premix (Bioneer Inc., Daejeon, Korea). cDNA obtained was amplified using a CFX96™ Real-Time System (Takara Inc., Otsu, Japan) with the following primers: collagen I forward, 5'-TCCCCAGCCACAAAGAGTCTACA-3' and reverse, 5'-GTGATTGGGTGGGATGTCTTCGTC-3'; tropoelastin forward, 5'-AAAGCAGCAGCAAAGTTCGG-3' and reverse, 5'-ACCTGGGACAACTGGAATCC-3'; MMP-1 forward, 5'-GGAGGGGATGCTCATTTTGATG-3' and reverse, 5'-TAGGGAAGCCAAAGGAGCTGT-3'; MMP-3 forward, 5'-CCTGCTTTGTCCTTTGATGC-3' and reverse, 5'-TGAG TCAATCCCTGGAAAGTC-3'; TIMP-1 forward, 5'-TTCGT GGGGACACCAGAAGTCAAC-3' and reverse, 5'-TGGACA CTGTGCAGGCTTCAGTTC -3'; and GAPDH forward, 5'-GAG TCAACGGATTTGGTCGT-3' and reverse, 5'-TTGATTTTGG AGGGATCTCG-3'.

The PCR profiles were as follows: 94˚C for 5 min, 30 cycles for 1 min at 94˚C, 1 min at 55-60˚C, and 1 min at 72˚C, with a final extension step of 72˚C for 10 min. The resulting PCR products were visualized by electrophoretic separation on 1.5% agarose gels containing 1 μg ethidium bromide/ml. The gel samples were prepared by mixing 20 μl of reaction mixture with loading buffer and were then separated by electrophoresis for 20 min at 100 V before being visualized with a ChemiDoc XRS system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Each band was densitometrically quantified by image analysis and controlled vs. GAPDH intensity.

Statistical analysis. One-way ANOVA followed by Dunnett's T3 test was used to assess statistical significance with thresholds of $P<0.05$, $P<0.01$ and $P<0.001$ indicating significant and highly significant differences, respectively.

Results

Extract of the mycelium of T. matsutake does not alter the expression of type I collagen in human fibroblasts. To determine whether the extract of the mycelium of *T. matsutake*t exerts a proliferative effect on human fibroblasts, we treated human fibroblasts with extract of the mycelium of *T. matsutake* at concentrations of 1-100 μg/ml for 72 h. Cell proliferation was determined by MTT assay; the results revealed that the extract of the mycelium of *T. matsutake* did not increase cell proliferation (Fig. 1A). Collagens are necessary proteins found abundantly in the dermis and are normally produced by fibroblasts. Thus, we investigated the expression of collagen I in fibroblasts treated with extract of the mycelium of *T. matsutake*. As shown in Fig. 1B, the mRNA and protein expression of collagen I remained unaltered following treatment with various doses of the extract of the mycelium of *T. matsutake* treatment. This result suggests that the mycelium of *T. matsutake* does not have the potential to promote the synthesis of collagen I.

Extract of the mycelium of T. matsutake decreases the mRNA and protein expression of MMP-1 and MMP-3 in human fibroblasts. We analyzed the expression of MMPs in fibroblasts treated with extract of the mycelium of *T. matsutake*, and found that it decreased the mRNA and protein expression of MMP-3 in the cells treated with 100 μg/ml of the extract. Furthermore, we found that treatment with extract of the mycelium of *T. matsutake* clearly decreased the mRNA and protein expression of MMP-1 in a dose-independent manner (Fig. 1C).

Extract of the mycelium of T. matsutake increases the expression of TIMP-1 and tropoelastin in human fibroblasts. Our results demonstrated that the extract of the mycelium of *T. matsutake* decreased the expression of MMP-1. To evaluate the anti-wrinkle effects of the mycelium of *T. matsutake* in human fibroblasts, we determined the protein expression of TIMP-1 in the fibroblasts treated with various concentrations of extract of the mycelium of *T. matsutake*. TIMPs are known as inhibitors of MMP and are produced by fibroblasts. We found that the expression of TIMP-1 increased in the fibroblasts treated with extract of the mycelium of *T. matsutake*; the increase in the expression of TIMP-1 was clearly evident at the concentration of 1 μg/ml of the extract (Fig. 2A). In addition, we evaluated the effects of the extract of the mycelium of *T. matsutake* on the mRNA expression of TIMP-1 in human fibroblasts. As shown in Fig. 2A, extract of the mycelium of *T. matsutake* markedly increased the mRNA expression of TIMP-1 in the cells treated with 100 μg/ml of the extract.

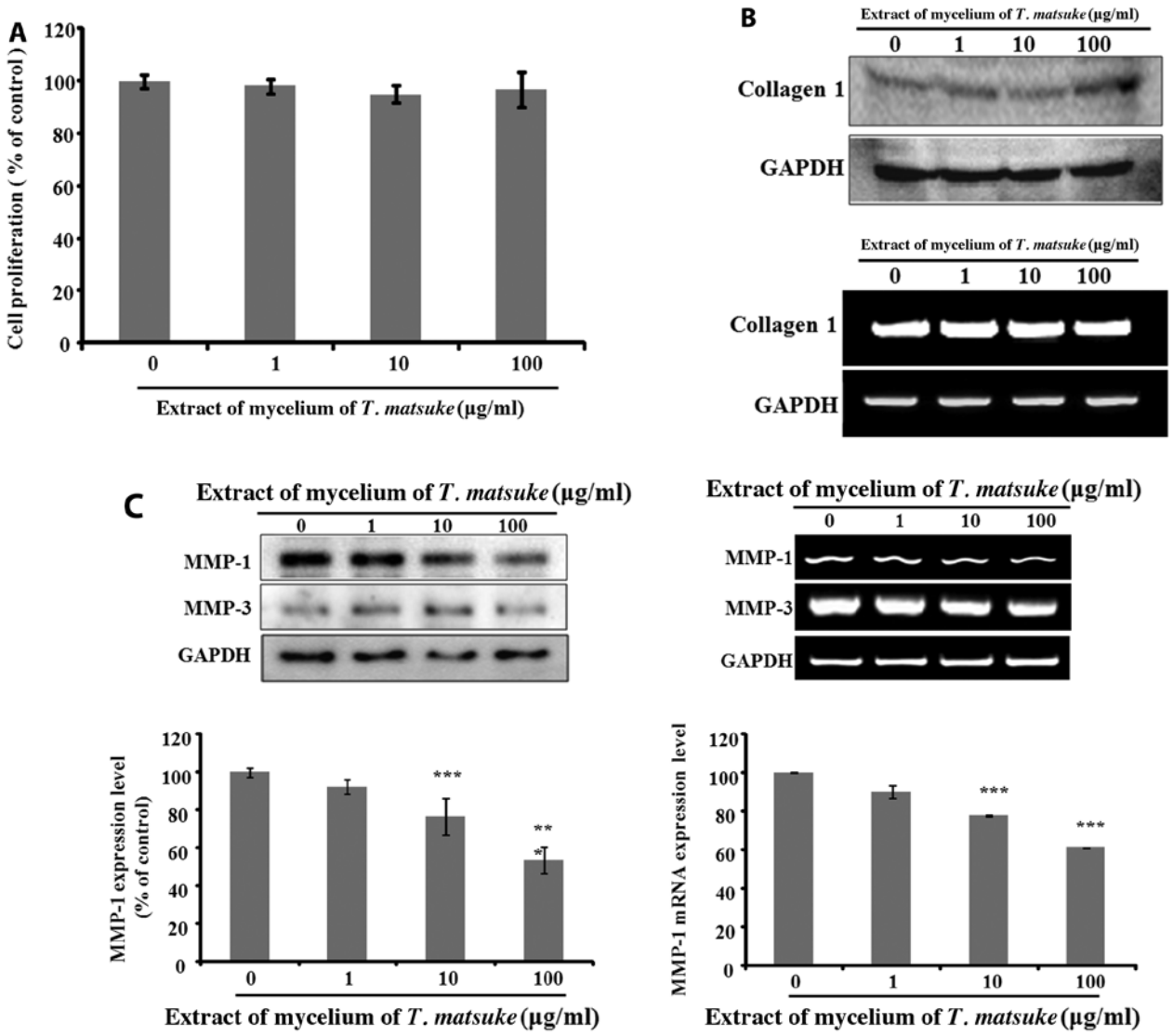

Figure 1. Matrix metalloproteinase (MMP)-1 expression is decreased by the mycelium of *Tricholoma matsutake* (*T. matsutake)* in human fibroblasts but collagen I levels are not altered. After being serum-starved for 24 h, human fibroblasts were treated with extract of the mycelium of *T. matsutake* at the indicated concentrations for 72 h in serum-conditioned medium. (A) Cell proliferation was analyzed by MTT assay. Follwoing serum starvation, human fibroblasts were treated with extract of the mycelium of *T. matsutake* (1-100 μg/ml) for 72 h. (B) Cultured cells were harvested for the detection of collagen I mRNA and protein expression by RT-PCR and western blot analysis, respectively. (C) Cells treated with extract of the mycelium of *T. matsutake* were lysed for the detection of MMP-1 and MMP-3 mRNA and protein expression by RT-PCR and western blot analysis, respectively. Equal protein and mRNA loadings were confirmed by reaction with GAPDH antibody and gene. These results are representative of 3 independent experiments. Values shown are the means ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs. control.

We then examined the expression of tropoelastin in fibroblasts treated with the extract of the mycelium of *T. matsutake*. Tropoelastin is a soluble precursor of elastin. To quantitatively assess tropoelastin expression, tropoelastin mRNA expression was analyzed by RT-PCR. The results revealed that the extract of the mycelium of *T. matsutake* increased the mRNA expression of tropoelstin compared to the untreated cells (Fig. 2B). To verify our results, we evaluated tropoelastin expression by immunofluorescence staining. We found that the expression of tropoelastin was increased in the cells treated with extract of the mycelium of *T. matsutake* compared to the untreated cells (Fig. 2C). Thus, these results support our observation of the increased expression of TIMP-1 and tropoelastin in fibroblasts treated with extract of the mycelium of *T. matsutake*; the increase in the expression of TIMP-1 was mediated by the downregulation in the expression of MMPs in fibroblasts.

Extract of the mycelium of T. matsutake inhibits elastase activity in human fibroblasts. Fibroblast elastase is a metalloproteinase that has been reported to play a role in the degradation of elastin (23). Elastase is inhibited by metal chelating agents, such as EDTA, as well as 1,10-phenanthroline and phosphoramidon as a metalloproteinase inhibitor (22). To determine whether the extract of the mycelium of *T. matsutake* exerts an inhibitory effect on human fibroblast-derived elastase, we treated the cells with various concentrations (0.1-100 μg/ml) of extract of the mycelium of *T. matsutake*. We determined elastase activity in human fibroblasts using STANA as a substrate and phosphoramidon. As a result, the extract of the mycelium of *T. matsutake* exerted an inhibitory effect on human fibroblast-derived elastase in a dose-dependent manner; the most potent inhibitory effects of the extract of the mycelium of *T. matsutake* (81.4±3.92%) were observed at a concentration

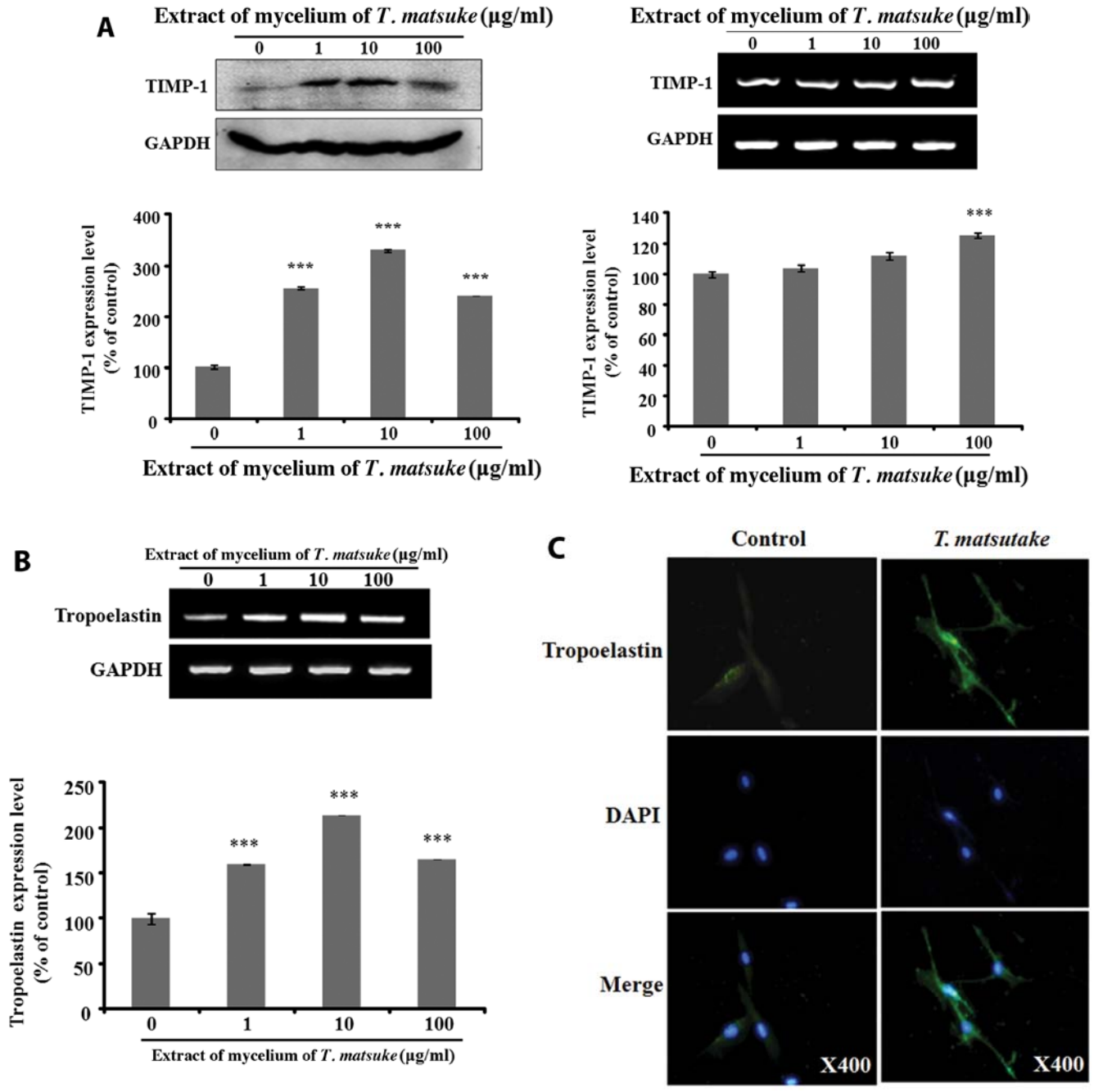

Figure 2. Treatment with the extract of the mycelium of *Tricholoma matsutake* (*T. matsutake)* increases the expression of the tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) and tropoelastin in human fibroblasts. Following serum starvation, human fibroblasts were treated with extract of the mycelium of *T. matsutake* (1-100 μg/ml) for 72 h. (A) Cultured cells were harvested for the detection of the mRNA and protein expression of TIMP-1 by RT-PCR and western blot analysis, respectively. (B) Tropoelastin protein expression was analyzed by western blot analysis. Equal protein and mRNA loadings were confirmed by reaction with GAPDH. The results are representative of 3 independent experiments. Values shown are the means ± SEM. $^{***}p<0.001$ vs. controls. (C) Cells were prepared by adding $5x10^4$ cells to glass coverslips followed by incubation for 24 h. Following serum starvation, the cells were treated with extract of the mycelium of *T. matsutake* (100 μg/ml). The samples were prepared for immunofluorescence staining. Untreated cells and cells treated with extract of the mycelium of *T. matsutake* were incubated with antibodies specific for tropoelastin and stained with fluorescein isothiocyanate (FITC)-conjugated secondary antibody. In addition, 4,6-diamino-2-phenylidole (DAPI) staining was performed. Fluorescence was detected under a fluorescence microscope. The specimens were photographed using DP Controller software (x400 magnification).

of 100 μg/ml, and those of phophoramidon (positive control, 89.6±7.74%) at a concentration of 10 μM (Fig. 3). These results indicate that the mycelium of *T. matsutake* prevented the degradation of elastin by inhibiting elastase activity.

Treatment with extract of the mycelium of T. matsutake inhibits TPA-induced MMP-1 expression in human fibroblasts. To confirm the inhibitory effects of the extract of the mycelium of *T. matsutake* (100 μg/ml) on MMP-1 expression in human fibroblasts, we examined the expression of MMP-1 in fibroblasts treated with TPA prior to treatment with extract of the mycelium of *T. matsutake*. TPA induced an increase in the mRNA and protein expression of MMP-1 in a dose-dependent manner (Fig. 4A). The increase in the mRNA and

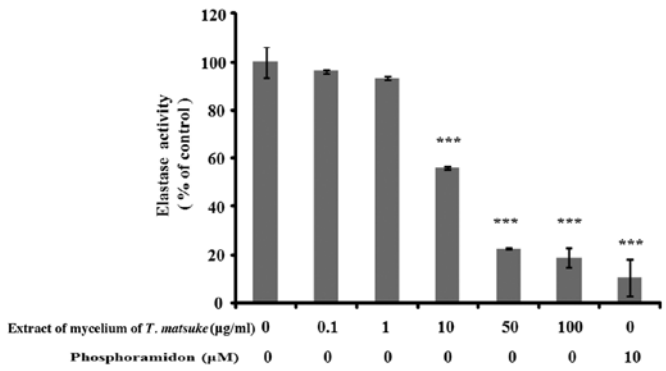

Figure 3. Elastase activity was inhibited by extract of the mycelium of *Tricholoma matsutake* (*T. matsutake)* in a dose-dependent manner in human fibroblasts. Cultured cells were harvested for the detection of elastase activity, as described in the Materials and methods. These results are representative of 3 independent experiments. Values shown are the means ± SEM. ***p<0.001 vs. controls.

protein expression of MMP-1 induced by TPA (60 nM) was abrogated by MMP inhibitor I (decrease of 39.7% compared to the level observed with 60 nM TPA). In addition, treatment with extract of the mycelium of *T. matsutake* (100 μg/ml) significantly decreased MMP-1 protein expression by 26.5% compared to the level observed with 60 nM TPA (Fig. 4B). As shown in Fig. 4C, treatment with extract of the mycelium of *T. matsutake* (100 μg/ml) inhibited TPA-induced MMP-1 mRNA expression in human fibroblasts by 17.2% compared to the level observed with 60 nM TPA.

Induction of MMP-1 expression by TPA is mediated through p38, and is inhibited by extract of the mycelium of T. matsutake. Previous studies have elucidated the roles of distinct mitogen-activated protein kinase (MAPK) pathways in the regulation of MMP-1 (24). In addition, it has been reported that the induction of MMP-1 expression by okadaic acid requires the simultaneous activation of the extracellular signal-regulated kinase (ERK)1/2, Jun N-terminal kinase (JNK) and/or p38 pathways (24,25). To determine the possible signaling pathway through which TPA increases the expression of MMP-1, we examined whether the induction of MMP-1 expression by TPA occurs through the phosphorylation of p38 (p-p38). The fibroblasts were treated with TPA alone or in combination with p38 inhibitor or extract of the mycelium of *T. matsutake*, and the levels of p-p38 were determined by western blot analysis. The expression of p-p38 protein was increased in the TPA-treated cells (Fig. 5A). We evaluated the role of p38 in the TPA-induced expression of MMP-1 in human fibroblasts by blocking p-p38 using inhibitors. It has been reported that the upregulation of MMP-1 expression by p38 is activated by diverse stimuli (26). As shown in Fig. 5B, the increase in the expression of p-p38 induced by TPA (60 nM) was inhibited by SB203580 (SB), a specific inhibitor of p38. Of note, the extract of the mycelium of *T. matsutake* had an inhibitory effect on p-p38 expression induced by TPA. In parallel, the expression of p-p38 induced by TPA was potently inhibited by pre-treatment with extract of the mycelium of *T. matsutake* in combination wtih SB, indicating that the activation of p-p38 may have equal effects on MMP-1 expression (Fig. 5C). In addition, the induc-

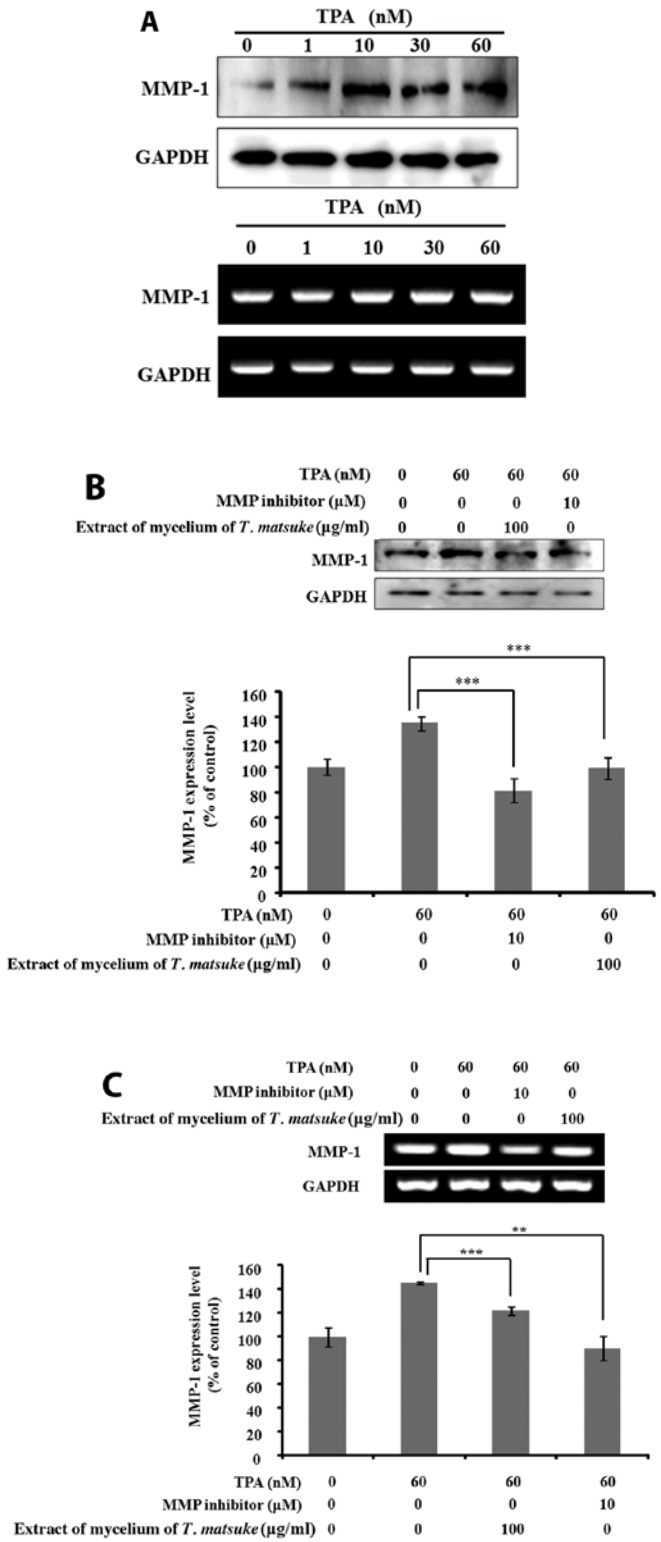

Figure 4. Extract of the mycelium of *Tricholoma matsutake* (*T. matsutake)* inhibits the 2-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced expression of matrix metalloproteinase-1 (MMP-1) expression. (A) Following serum starvation for 24 h, human fibroblasts were treated with TPA (1-60 nM) for 24 h. Cultured cells were harvested for the detection of the mRNA and protein expression of MMP-1 by RT-PCR and western blot analysis, respectively. Human fibroblasts were treated with TPA (60 nM), MMP inhibitor I (10 μM) and extract of the mycelium of *T. matsutake* (100 μg/ml) alone or in combination for 24 h. MMP-1 protein and mRNA expression was analyzed by (B) western blot analysis and (C) RT-PCR, respectively. Equal protein and mRNA loadings were confirmed by reactions with GAPDH. The results are representative of 3 independent experiments. Values shown are the means ± SEM. **p<0.01, ***p<0.001 vs. TPA-treated cells.

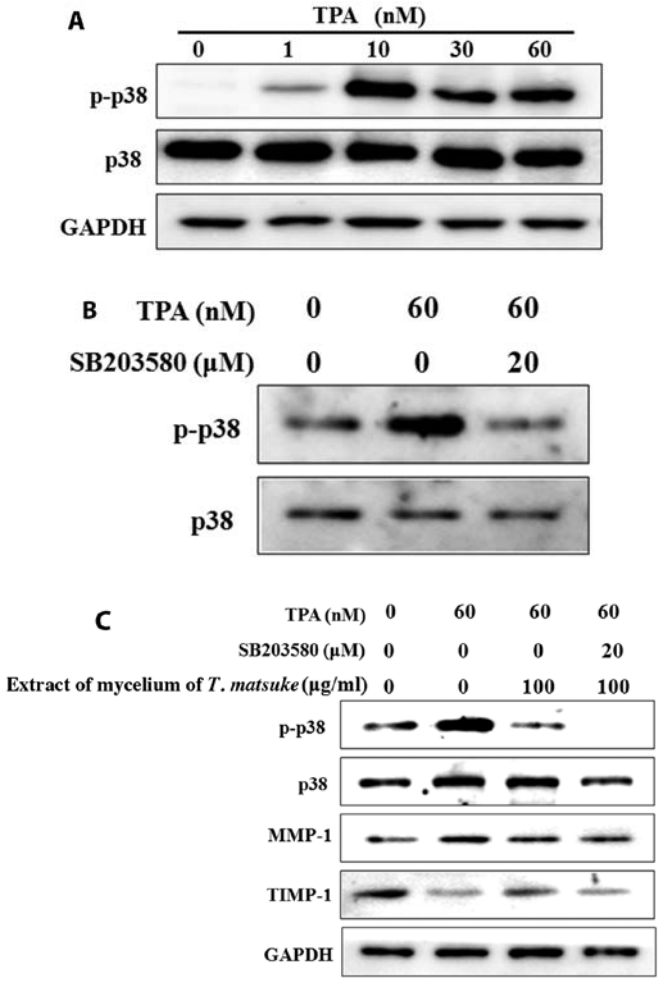

Figure 5. Inhibitory effects of extract of the mycelium of *Tricholoma matsutake* (*T. matsutake)* on matrix metalloproteinase-1 (MMP-1) activation are mediated by p38 mitogen-activated protein kinase (MAPK) in human fibroblasts. Human fibroblasts were treated with extract of the mycelium of *T. matsutake* and 2-*O*-tetradecanoylphorbol-13-acetate (TPA) at the indicated concentrations. Cells were lysed, and the level of phosphorylated (p)-p38 was determined by western blot analysis with phospho-specific antibodies. Protein loading was evaluated using antibodies against total p38. (A) Human fibroblasts were treated for 24 h with TPA (1-60 nM) at the indicated concentrations. (B) Human fibroblasts were treated with specific inhibitor of p-p38 [SB203580 (SB 20 μM)] for 2 h, and then treated with 60 nM TPA alone or in combination for 24 h. (C) Following serum starvation for 24 h, human fibroblasts were incubated for 24 h with TPA (60 nM) alone or in combination. Where indicated, cell were treated for 2 h with extract of the mycelium of *T. matsutake* (100 μg/ml) and SB203580 (SB 20 μM). Cells were harvested and the expression levels of p-p38, total p38, MMP-1 and tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) were detected by western blot analysis. Equal protein loadings were confirmed by reactions with GAPDH antibody. The results are representative of 3 independent experiments.

tion of MMP-1 expression by TPA was potently inhibited by pre-treatment with extract of the mycelium of *T. matsutake* in combination with SB, whereas the expression of TIMP-1 which was inhibited by TPA was restored by treatment with the extract (Fig. 5C). These results demonstrated that SB and the extract of the mycelium of *T. matsutake* decreased the efficacy of TPA in increasing MMP-1 expression. This finding suggests that the TPA-induced expression of MMP-1 may be regulated through the p38 pathway, and that the mycelium of *T. matsutake* has an abrogating effect on p38 activation induced by TPA.

Discussion

The extract of the mycelium of *T. matsutake*, a biopharmaceutical polysaccharide component, has a wide range of medicinal effects, including reduced oxidation, inhibition of inflammation and anticancer properties (16,19,27,28). However, the anti-aging effects of the mycelium of *T. matsutake* in human fibroblasts are not yet well known, and to the best of our knowledge, its effects on MMP-1 and TIMP-1 expression have not been reported to date. In this study, we investigated the potent wrinkle inhibitory effects of the extract of the mycelium of *T. matsutake* and its effects on the basal and TPA-induced expression of MMP-1 in human fibroblasts. We demonstrated that the extract of the mycelium of *T. matsutake* is an anti-wrinkle agent that acts by inhibiting the basal and TPA-induced expression of MMP-1 and increasing the expression of TIMP-1 and tropoelastin.

The remodeling of the extracellular matrix is essential for processes, such as skin aging, wound healing and fibrosis. Collagen and elastic fibers in the ECM network are related to skin aging symptoms, such as wrinkles, sagging and looseness. The degradation of collagen and elastin fibers in the skin is mainly caused by the expression of MMP-1, MMP-3 and elastase (29,30). MMP activity is regulated by TIMPs. TIMPs are naturally produced proteins that are important regulators of ECM turnover (30,31). The ratio between MMPs and TIMPs plays an essential role leading to ECM remodeling. An imbalance in the MMP/TIMP ratio has been shown to be involved in various diseases in humans (13,29). In this study, treatment of fibroblasts with extract of the mycelium of *T. matsutake* suppressed the basal levels of MMP-1 and MMP-3 expression (Fig. 1C). MMP-1 expression decreased significantly by 10±3.21, 23±0.68 and 39±0.46% in relation to the controls (untreated cells) following treatment with extract of the mycelium of *T. matsutake* at doses of 1, 10 and 100 μg/ml, respectively. On the other hand, the basal level of TIMP-1 expression increased in a dose-dependent manner following treatment with the extract (Fig. 2A). The basal level of TIMP-1 expression significantly increased by 154±2.42, 218±2.66 and 139±0.41% compared to the controls following treatment with extract of the mycelium of *T. matsutake* at doses of 1, 10 and 100 μg/ml, respectively. The effects of the extract of the mycelium of *T. matsutake* occurred in a dose-dependent manner and were not cytotoxic. The progression of skin aging caused by harmful agents in the environment is accompanied by both inflammatory reactions and oxidative damage (16,32). Previously, the extract of the mycelium of *T. matsutake* was shown to exert antioxidant effects, including an antioxidant component that enhances skin resistance to external factors and inhibits skin oxidation (33). In our study, we demonstrated that the decrease in MMP-1 expression and the increase in TIMP-1 expression may be mediated by the anti-inflammatory and antioxidant effects of the mycelium of *T. matsutake* in human fibroblasts.

The general age-associated characteristic in both internal and external processes is the loss of normal elastic fiber functions through the degradation of elastin, as exhibited in the wrinkling and sagging of the skin (3,4). The proteolytic degradation of elastin results in the formation of elastase as MMP-1 is the endogenous inhibitor present in the dermis. It

has been reported that skin fibroblast elastase is involved in the metabolism of elastic fiber during aging (6,34). Enhanced skin fibroblast elastase has been demonstrated to cause the loss of skin elasticity and is the primary component of phosphoramidon, which is known to be a typical elastase inhibitor, and significantly inhibits fibroblast elastase activity by more than 89.6% (35). However, phosphoramidon exhibits poor penetration through the skin, including the hydrophilic rhamnose residue. Researchers have focused on overcoming these issues, and have investigated N-phenetylphosphonyl-leucyl-tryptophane (NPLT), which has similar potency to the inhibitory effects of phosphoramidon (22,36). On the other hand, tropoelastin, a soluble elastin molecule and major structural component of microfibrils, is produced by fibroblasts and is a protein of the ECM (2). The proteolysis of microfibrils is caused by degradation emzymes, such as elastases and MMPs (10). It has previously been reported that ellagic acid and tannic acid, which are known polyphenols, may be useful in preventing the proteolytic degradation of dermal elastic fibers and in enhancing the expression of tropoelastin (37). We hypothesized that the extract of the mycelium of *T. matsutake* would have an effect on cellular elastase activity and used it as an inhibitor of fibroblast elastase to examine this possibility. In addition, we investigated the expression of tropoelastin in fibroblasts treated with extract of the mycelium of *T. matsutake*. Our results revealed that the extract of the mycelium of *T. matsutake* significantly decreased elastase activity in a dose-dependent manner. Moreover, as shown in Fig. 2C, treatment with extract of the mycelium of *T. matsutake* increased tropoelastin expression. It is clear that the elastase inhibitory effects of extract of the mycelium of *T. matsutake* in fibroblasts are more potent than those of phosphoramidone. This suggests that the extract of the mycelium of *T. matsutake* may be a reliable anti-aging agent for use in the cosmetics industry.

Increased MMP-1 levels have been shown to be involved in the metastasis of several types of tumor (38); moreover, they facilitate skin wrinkling following exposure to UV irradiation (39). It has also been reported that MMP-1 expression is increased by several stimuli, such as cytokines, growth factors, tumor-promoting agents and UV irradiation (40). TPA, a well-known tumor-promoting agent, is a protein kinase C (PKC) activator and can increase MMP-1 production in various types of cells through the activation of PKC-dependent and/or -independent signaling pathways (41-43). The molecular mechanisms responsible for TPA-induced MMP-1 expression involve the stress-activated MAPK pathway, as well as c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 (44). In this study, to investigate the correlation between p38 inhibition and MMP-1 reduction, fibroblasts were treated with SB203580 (a p38 inhibitor) prior to treatment with extract of the mycelium of *T. matsutake* or TPA and the expression of p-p38 was evaluated by western blot analysis. Our results revealed that the level p-p38 was increased in the TPA-treated fibroblasts (Fig. 5A). Our results also demonstrated that pre-treatment with SB203580 and extract of the mycelium of *T. matsutake* alone or in combination inhibited the activation of p38 and decreased TPA-induced MMP-1 expression levels (Fig. 5C). However, the expression of TIMP-1 was increased under the same conditions (Fig. 5C): the inhibition of p38 and the suppression of MMP-1 expression in the human fibroblasts was observed concurrently, indicating a possible mechanism of action of the extract of the mycelium of *T. matsutake*.

The increased expression of MMP-1 has been shown to correlate with several other factors, such as the ERK1/2, JNK, Raf-1, MKK3, AP-1 and ETS transcription factors (45,46). Previous studies have found that these pathways are involved in the regulation of MMP-1 expression in fibroblasts (45,47). However, we did not examine the correlation between MMP-1 expression and phosphorylated PKC levels, phosphorylated ERK levels or phosphorylated JNK levels following treatment with extract of the mycelium of *T. matsutake* and specific inhibitors, such as staurosporine (PKC inhibitor), PD98059 (MEK inhibitor), or SP600125 (JNK inhibitor). Therefore, to fully elucidate the role of these pathways in the inhibition of the TPA-induced production of MMP-1 by the extract of the mycelium of *T. matsutake* in fibroblasts, further studies are required.

In conclusion, our results suggest that the treatment of human fibroblasts with extract of the mycelium of *T. matsutake* decreases the basal levels and the TPA-induced MMP-1 expression in a dose-dependent manner. In addition, extract of the mycelium of *T. matsutake* decreased elastase activity in a dose-dependent manner. These data suggest that extract of the mycelium of *T. matsutake* may be used as an effective biomaterial in-aging treatments that can obstruct the degradation of the dermal ECM by inhibiting elastase activity and MMP-1 expression.

Acknowledgements

This study was supported by Biomedical Science Scholarship Grants, Department of Medicine, Chung-Ang University in 2012.

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