Influence of Gungguitang-gamibang on the Regulation of Melanogenesis through JNK Signaling Pathway in B16 Melanoma Cells

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Gunggui-tang has been used for the therapy of blood disorders in Hangbang medicine for long time. Also, *Glycyrrhiza uralensis* has been used for deficientblood patterns with an irregular pulse or palpitations, coughing and wheezing, and heat or cold in the lungs. Melanogenesis is a physiological process resulting in the synthesis of melanin pigments. We investigated whether the water extract of Gunggui-tang plus G. uralensis inhibited melanogenesis in B16 melanoma cells. Because the molecular events connecting the regulation in tyrosinase activity remain to be elucidated, we also aimed to determine whether Gunggui-tang gamibang(GTG) affects tyrosinase at the gene activation level in the cells. First, we showed that GTG inhibited the tyrosinase promoter activity and further, down-regulated the tyrosinase protein activity in α-melanocyte-stimulating hormone (α-MSH)-treated B16 melanoma cells. GTG also resulted in a decrease of melanin content in MSH-induced melanogenesis, indicating that GTG may be a useful drug in studying the regulation of melanogenesis. The pretreatment of GTG significantly prevented phosphotransferase activity of c-Jun N-terminal kinase (JNK1) and transcriptional activation of activating protein-1 (AP-1) in MSH-treated B16 melanoma cells. These findings indicate that GTG inhibits melanogenesis of B16 melanoma cells via suppression of phosphotransferase activity of JNK1 and transcriptional activation of AP-1.

Key words : Gunggui-tang-gamibang, melanogenesis, JNK, B16 melanoma cell

Introduction

Over the past decade there has been a resurgence of interest in botanical products for their medicinal uses. Gunggui-tang(GTG) is a basic prescription for the blood disorder in Hanbang medicine. *Glycyrrhiza uralensis* Fisch is one of the important traditional herb medicines, and the main known effective ingredient is glycyrrhizin[1]. This has been commonly used for deficient spleen patterns, deficient blood patterns with an irregular or intermittent pulse or palpitations, coughing and wheezing, raw for carbuncles, sore throat arising from fire poison, painful spasms in the abdomen and legs, and heat or cold in the lungs[2]. The water extract of *G. uralensis* was used with combination of a mixture of crude extracts from other medicinal plants to develop and apply of new medicines. However, the study on melanogenesis in B16 melanoma cells by this prescriptions was still not investigated.

Melanogenesis is a physiological process resulting in the synthesis of melanin pigments, which play a crucial protective role against skin photocarcinogenesis[3]. Melanin is a mixture of pigmented bipolymers synthesized by specialized cells such as melanocytes and melanoma cells[4]. Melanin synthesis within melanosomes and their distribution to keratinocytes within the epidermal melanin unit determine skin pigmentation. Among the agents secreted by keratinocytes upon ultraviolet-B treatment, α-melanocyte-stimulating hormone (MSH) is one of the most potent activators of melanogenesis. Addition of MSH in cultured human melanocytes[5] or in melanoma cells[6] stimulates melanization. It has been shown that MSH-induced melanogenesis in mouse melanoma cells occurs by an increase of intracellular cAMP content, an accumulation of tyrosinase mRNA, and a stimulation of tyrosinase activity[7].

Melanin biosynthesis consists in a cascade of enzymatic and spontaneous reactions that converts tyrosine to melanin pigments[8]. The initial and rate-limiting step in melanin synthesis, the hydroxylation of tyrosine to DOPA, is controlled by tyrosinase that is the key enzyme in this process. Therefore,

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the melanin synthetic pathway is blocked without enzymatic action of tyrosinase. Tyrosinase expression is usually regulated at the transcriptional level, depending on both positive and negative regulatory elements working in concert to modulate promoter activity. However, melanin-specific transcription factors have not yet been identified, and only indirect evidence links the microphthalmia gene product to the regulation of the tyrosinase promoter by cAMP[9]. Englaro et al. (1998)[10] previous reported that mitogen-activated protein (MAP) kinase pathway could be involved in the regulation of melanoma cell differentiation and, more precisely, that the activation of extracellular signal-regulated kinases (ERKs) would be a required event in the induction of melanogenesis by cAMP-elevating agents. The c-Jun N-terminal kinase (JNK) is a superfamily of the MAP kinase family of serine/threonine kinase and often plays important regulatory roles in signal transduction[11,12]. The products of the Jun and Fos family genes are components of the transcription factor, activating protein-1 (AP-1)[13]. Transcriptional activity of AP-1 is regulated by the phosphorylation status of c-Jun[14]. Phosphorylation of the c-Jun N-terminal domain is catalyzed by the JNK[15]. Here, we report that glycyrrhizae antagonizes the effects of MSH, triggering a decrease of tyrosinase expression through a mechanism that might involve JNK signaling pathway.

Concerns of changes in skin color are frequently raised for medical or cosmetic reasons. Hyperpigmentation disorders are often treated with hydroquinones, retinoids, and tyrosinase inhibitors, but results of such treatments are sometimes disappointing[16]. In the present study, we tested the effects of the water extract of GTG(GTGWE) on the melanogenesis of B16 melanoma cells. We found that GTGWE inhibited melanogenesis through the suppressions of tyrosinase activity, phosphotransferase activity of JNK1, and transcriptional activation of AP-1. Therefore, we suggest that GTGWE may serve as an alternative treatment for depigmentation.

Materials and Methods

1. Cell culture

The mouse B16 melanoma cellswere acquired from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 medium containing 2 mM L-glutamine, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Biofluids, Rockville, MD) in a humidified atmosphere chamber containing 5% CO_2 at 37C .

2. Preparation of GTGWE

The plant sample was obtained from the Oriental drug store, Bohwa-dang (Iksan, Korea). Botanical identification was confirmed at the Herbarium of the College of Oriental Medicine, and the specimen was deposited with voucher number OM-PH-006. An aqueous extract of GTGWE was prepared by decocting the dried prescription of herbs with boiling distilled water for 3 h. The decoction was filtered, lyophilized, and kept at 70C. The yield of dried extract from starting crude materials was about 10%.

3. MTT assay

Growth of B16 melanoma cells in the presence of GTGWE was determined by methylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT; Sigma Co., Saint Louise, MO) assay. Cells were seeded in 96 well microculture plate at 2 x 10^3 cells per well and allowed to adhere overnight. Cells were exposed to 10, 50, 100, and 200 μg/ml of GTGWE for 24 h and then MTT assays were performed. MTT was prepared at a concentration of 5 mg/ml in sterile PBS and a 20 $\mu\ell$ aliquot of the stock solution was added to each well. After 3 h of incubation at 37C, 100 $\mu\ell$ of ethanol-DMSO (1:1 mixture solution) was added to each well in order to dissolve the formazan crystals. Optical densities were measured at 540 nm using a 96 well Multiscanner (Dynatech Instruments, Torrance, CA). A well containing RPMI 1640 medium, MTT, and extration buffer in the absence of B16 melanoma cells was used as the blank. The results obtained were calculated from 4 wells and presented as percentage of control values.

4. Tyrosinase promoter activity assay

B16 melanoma cells were plated in 6-well dishes and transfected with 2 μg of tyrosinase promotor reporter plasmid per well, using LipofectAMINE system according to the manufacturer's instructions (Life Technologies, Inc.). Cells were co-transfected with 0.5 μg of the β-galactosidase reporter vector pCMVβ(Promega) to control the variability in transfection efficiency. Eighteen hours after transfection, cells were further incubated for 12 h in the absence or presence of 10, 50, 100, and 200 μg/ml of GTGWE. For the luciferase assays, cells were washed with PBS, lysed with reporter lysis buffer, and processed to generate cell supernatant and pellet fractions according to the manufacturer's recommendations (Promega Luciferase Assay System). Soluble extracts were harvested and assayed for luciferase and β-galactosidase activity. All transfections were repeated at least three times using different preparations.

5. Tyrosinase activity assay

Tyrosinase activity was determined by a method of

Martinez-Esparza et al. (1998)[17]. For this experiment, cells were preincubated with 50 ㎍/ml of GTGWE for 30 min and then treated with 10 nM α-MSH for an additional 72 h at 37C. Where indicated, cells were also treated with α-MSH alone in a dose-dependent manner. The cells were then washed twice with ice-cold PBS and lysed in lysis buffer (1% Triton X-100, 10 mM sodium phosphate, pH 7.0) supplemented with protease inhibitors (0.1 mM sodium orthovanadate, 2 ㎍/ml aprotinin, 2 ㎍/ml leupeptin, and 100 ㎍/ml PMSF) for 30 min on ice. Lysates were centrifuged at 9000 x g for 15 min to remove insoluble material. Protein concentration was determined by the Bradford method (Bio-Rad) using BSA as a standard. A 50 l of samples was mixed with 100 l of 100 mM sodium phosphate (pH 7.0) at 30 C for 5 min, and then added 50 ㎕ of 100 mM catechol. Activity was assayed at 405 nm for 1 h using Perkin Elmer Spectrophotometer.

6. Melanin content determination

The melanin content was determined according to modification of the procedure described by Hosoi et al. (1985) [18]. Briefly, B16 melanoma cells were seeded in 24-well plate at 3 x 10^4 cells per well, preincubated with 50 ㎍/ml of GTGWE for 30 min and then treated with 10 nM MSH for an additional 72 h at 37C. Where indicated, cells were also treated with MSH alone in a dose-dependent manner. The cells were harvested, washed twice with ice-cold PBS, and solubilized in 1 ml of 1N NaOH/10% DMSO for 2 h at 80C. Lysates were centrifuged at 12,000 x g for 10 min. The absorbance of the supernatant was measured at 405 nm, and the values were compared to the standard curve obtained with synthetic melanin dissolved and diluted in 0.85 N KOH.

7. In vitro immunocomplex kinase assay

B16 melanoma cells were pretreated with 50 ㎍/ml of GTGWE for 30 min prior to stimulation with 10 nM α-MSH for an additional 6 h at 37C. The cells were harvested, washed twice with ice-cold PBS, and lysed in lysis buffer (1% Triton X-100, 10 mM Tris, pH 7.6, 50 mM NaCl, 2 g/ml aprotinin, 5 mM EDTA, 50 mM NaF, 0.1% 2-mercaptoethanol, and 0.1 mM sodium orthovanadate) for 30 min on ice. Lysates were centrifuged at 9,000 x g for 20 min and soluble fractions were incubated with 1 ㎍ of anti-JNK1 antibody (Santa Cruz Inc., Santa Cruz, CA) on ice for 3 h. A 100 l of a 10% Pansorbin (Calbiochem, La Jolla, CA) was added to the anti-JNK1 immunoprecipitates and further incubated on ice for 1 h. The immune complex was washed once with EB buffer and twice with PAN buffer (10 mM PIPES, pH 7.4, 0.1% aprotinin, 100 mM NaCl). The immunocomplex was mixed with 2 ㎍ of GST-c-Jun NT1-79 protein as a substrate in 30 ㎕ of the reaction buffer (2 μM cold ATP, 2 mM DTT, 20 mM $MgCl_2$, 2 μCi [γ32-p]-ATP, and 20 mM Tris-HCl, pH 7.5) at 30C for 20 min. This reaction was terminated by addition of 20 l 1X SDS sample buffer, and samples were separated on 12.5% SDS-PAGE. The gel was dried under a vacuum, and the phosphotransferase activity was visualized by autoradiography.

8. Electrophoretic mobility shift assay (EMSA)

B16 melanoma cells were pretreated with 50 ㎍/ml of GTGWE for 30 min prior to stimulation with 10 nM MSH for an additional 1 h at 37C. Nuclei from G. uralensis- and GTG-treated and untreated B16 melanoma cells were extracted according to the procedure described by Jeong and Jue (1997) [19]. The cells were washed twice with ice-cold PBS and lysed with hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM dithiothreitol, 10 ㎍/ml aprotinin, 20 μM pepstatin A, 100 μM leupeptin) on ice for 10 min. After centrifugation at 1,000 x g for 5 min, the nuclear pellets were resuspended in extraction buffer (20 mM Hepes, pH 7.9, 25% (v/v) glycerol, 0.4 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM dithiothreitol) and incubated on ice for 10 min. The nuclear proteins in the supernatant were recovered after centrifugation at 9,000 x g for 15 min, quantified by using a BCA protein assay kit (Sigma Co., St. Louis, MO), and used to carry out EMSA. To measure the activation of transcription factor AP-1, the probes had the following sequences: 5′-AAGGCGCTTGATGACTCAGCCGGAA-3′ 5′-AAGGTTCCGGCTGAGTCAT CAAGCG-3′. Two complementary strands of the oligonucleotides were annealed and labeled with [α-p32]-dCTP using a random primer labeling kit (Amersham Life Science, UK). Nuclear extracts were reacted with 5 ng of the radio-labelled AP-1 probe. The reaction was performed in the presence of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, and 4% glycerol (final volume: 25 ml) at room temperature for 30 min. The reaction products were then subjected to 4% SDS-PAGE. The gels were dried under vacuum for 1 h and exposed for autoradiography.

9. Data analysis

The values are shown as means ± standard deviations of the mean. Statistical differences between mean values were detected by Student's t-test. Differences were considered significant from the each control when the p value was less than 0.05.

Results

1. Effect of GTGWE on the viability of B16 melanoma cells

The established B16 melanoma cell line offers a model system with readily quantifiable markers of melanogenesis. To examine the direct cytotoxic effect of G. uralensis, cells were incubated with the concentrations of 10, 50, 100, and 200 μg/ml of GTGWE for 24 h. The effect of the exposure to increasing concentrations of GTGWE on the viability of B16 melanoma cells, as assessed by the MTT test, is shown in Fig. 1. GTGWE had no significant effect on the cell viability as well as morphological change by itself in all treatment groups compared to untreated group.

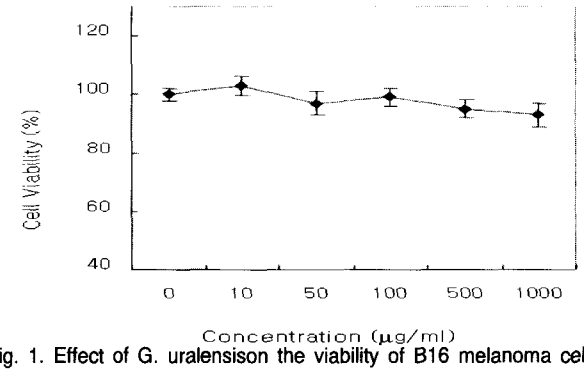

Fig. 1. Effect of G. uralensison the viability of B16 melanoma cells. Cells were treated with the concentrations of 10, 50, 100, 500, and 1,000 μg/ml of G. uralensis for 24 h. Cell viability by MTT assay was measured as described in Materialsand Methods. Results were expressed as the means ± S.D. of four experiments and presented as percentage of control values.

2. Effect of GTGWE on tyrosinase promoter activity in B16 melanoma cells

In mammalian melanocytes or melanoma cells, melanin synthesis is controlled by tyrosinase, the critical enzyme in the melanogenic pathway[20]. To investigate the regulation of tyrosinase gene expression in cultured B16 melanoma cells by the extract of GTGWE, cells were transiently transfected with tyrosinase promoter plasmid, cloned into a modified mammalian expression vector (pcDNA3.1) upstream of a firefly luciferase cDNA. Eighteen hours after transfection, cells were treated with the concentrations of 10, 50, 100, and 200 μg/ml of GTGWE for 12 h and the expression of tyrosinase promoter was determined using the luciferase assays. Tyrosinase promoter activity was significantly down-regulated in a dose-dependent manner in G. uralensis- and GTG-treated B16 melanoma cellscompared to untreated control cells (Fig. 2).

3. Effect of MSH on melanogenesis in B16 melanoma cells

Previous studies demonstrated that addition of α-MSH in cultured human melanocytes or in melanoma cells stimulates melanization[5,6]. We investigated the effect of α-MSH on tyrosinase activity in B16 melanoma cells. Cells were incubated for 72 h with the concentrations of 0.01, 0.5, 1.0, 5.0, and 10 nM of α-MSH. We found that tyrosinase activity was marked increased in a dose-dependent manner from 0.01 to 10 nM concentration (Fig. 3A). To clarify the influence of α-MSH on melanogenesis, we also measured melanin content after treatment of α-MSH. α-MSH-treated cells were increased in total melanin content and particularly at 10 nM increased by 270% approximately compared to untreated group (Fig. 3B). Tyrosinase activity was greater when treated with several concentrations of MSH for 72 h, than melanin content. It is considered that the role of tyrosinase is related to the initial phase, the hydroxylation of tyrosine to DOPA, in melanogenesis, whereas melanin formation is the final step passing through other numerous pathways and several stages.

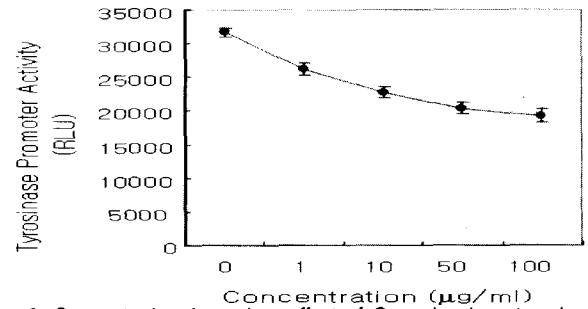

Fig. 2. Concentration-dependent effect of G. uralensison tyrosinase promoter activity in B16 melanoma cells. Cells were transfected with 2 μg tyrosinase promoter plasmid, cloned into a modified mammalian expression vector (pcDNA3.1). Eighteen hours after transfection, cells were treated with the concentrations of 1, 10, 50, and 100 μg/ml of G. uralensis for 12 h and the expression of tyrosinase promoter was determined using the luciferase assays.Results were expressed as the means ± S.D. of three experiments.

4. Effect of GTGWE on melanogenesis in α-MSH-treated B16 melanoma cells

Tyrosinase is the key enzyme in melanin biosynthesis, and a hallmark of differentiation in melanocytes and melanoma cells[21]. Therefore, in order to characterize the melanin synthesis and differentiative effects of G. uralensison B16 melanoma cells, we evaluated the influence of such treatment on tyrosinase enzyme activity. MSH treatment enhanced tyrosinase expression both at the protein and the mRNA level[22], thus we used this compound as negative control. B16 melanoma cells were preincubated for 30 min with 50 μg/ml of GTGWE and followed by addition of 10 nM α-MSH for 72 h. We found that B16 melanoma cells showed a marked increase in tyrosinase activity when treated with MSH, however, the addition of GTGWE significantly inhibited such α-MSH-induced tyrosinase enzyme activity. The administration of GTGWE alone decreased (0.5-fold) tyrosinase enzyme activity compared to untreated control level (Fig. 4A). Melanin is synthesized from the substrate tyrosine by the action of tyrosinase, and has a number of important roles including protection from ultraviolet light, the determination of phenotypic appearance, and absorption of toxic drugs[4,23]. In B16 melanoma cells treated with 10 nM MSH alone, the melanin

content was increased approximately 2.7-fold within 72 h compared to control group (Fig. 4B). GTGWE resulted in a decrease of melanin content in α-MSH-treated B16 melanoma cells as well as untreated cells, indicating that GTGWE sufficient to decrease the melanin content in the cells (Fig. 4B).

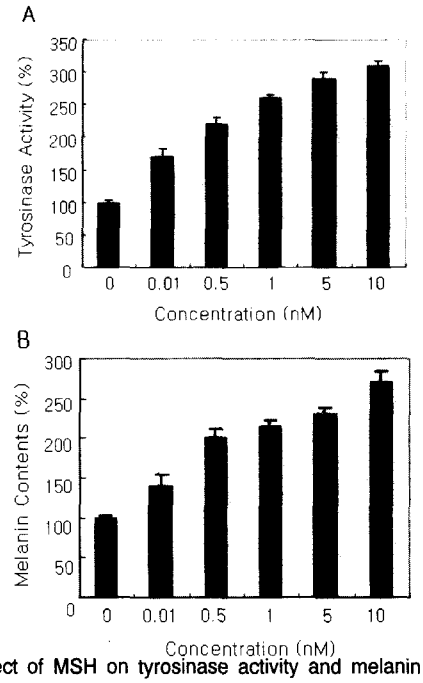

Fig. 3. Effect of MSH on tyrosinase activity and melanin content in B16 melanoma cells. The cells were incubated in the presence of various concentrations (0.01, 0.5, 1.0, 5.0, and 10.0 nM) of MSH for 72 h. Cells were harvested, washed twice with PBS, and lysed. Lysates were centrifuged and the absorbance of the supernatant fortyrosinase activity (A) and melanin content (B) was measured at 405 nm. Results were expressed as the means ± S.D. of three experiments and presented as percentage of control values.

5. Inhibition effects of GTGWE on α-MSH-mediated changes of JNK1 and AP-1 activations in B16 melanoma cells

Transcriptional activity of AP-1 is regulated by the phosphorylation state of the transactivation domain of c-Jun N-terminus[24]. Because this phosphorylation of c-Jun is catalyzed by JNK, a time point study was done on the effect of GTGWE on JNK1 activity in nuclear extract of B16 melanoma cells (Fig. 5A). The phosphotransferase activity of JNK1 was measured by an in vitro immunocomplex kinase assay and marked increased 6 h after treatment of 10 nM MSH alone (Fig. 5A). In addition, it showed that MSH-increased the activity of JNK1 was significantly reduced by pretreatment of 50 μg/ml of GTGWE(Fig. 5A). The c-Jun gene plays an important role in the transcriptional activity of Ap-1, since AP-1 binding to TRE is mediated via the binding sequence of the Jun protein. In this study we investigated the effect of GTGWE on AP-1 DNA binding activity in B16 melanoma cells. Fig. 5B shows that α -MSH after 1 h treatment enhanced AP-1 activation as expected, whereas the decrease in AP-1 activation in the cells treated with GTGWE. AP-1 activation by MSH was sharp inhibited in the presence of GTGWE consistent with the inhibition of JNK1 activation.

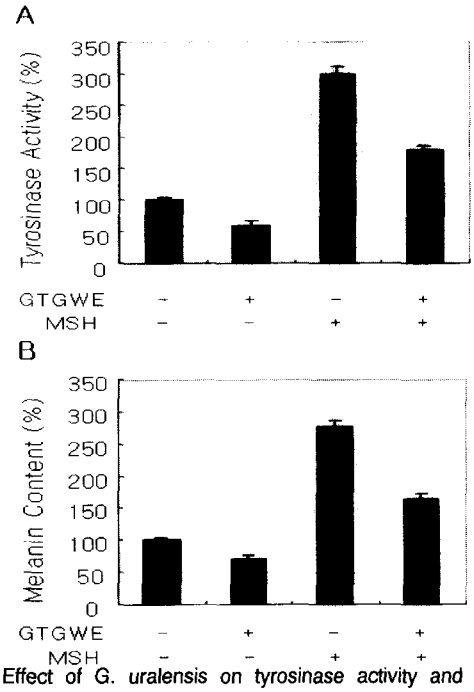

Fig. 4. Effect of G. uralensis on tyrosinase activity and melanin content in MSH-treated B16 melanoma cells. Cells were preincubated for 30 min with 50 mg/ml G. uralensis and followed by addition of 10 nM MSH for 72 h. Tyrosinase activity (A) and melanin content (B) were measured as in Fig. 3. Results were expressed as the means ± S.D. of three experiments and presented as percentage of control values.

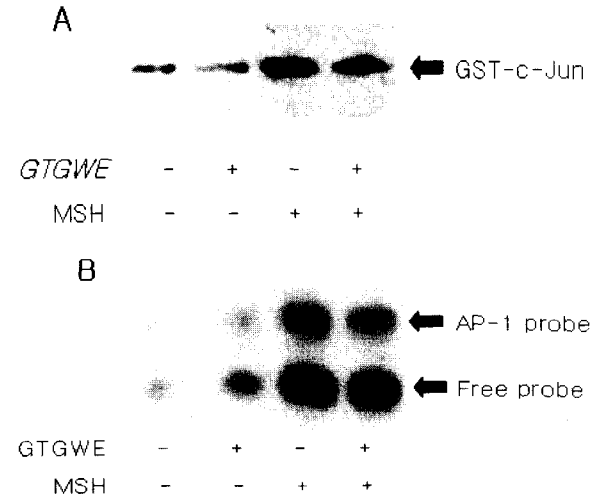

Fig. 5. Inhibition effect of G. uralensison MSH-mediated changes of JNK1 and AP-1 activations in B16 melanoma cells. (A) Cells were preincubated with 50 μg/ml G. uralensis for 30 min and followed by addition of 10 nM MSH for 6 h. Lysates were immunoprecipitated with anti-JNK1 antibody and immunocomplex was reacted with GST-c-Jun as a substrate 30 C for 20 min. Samples were separated on 12.5% SDS-PAGE and the phosphotransferase activity of JNK1was visualized by autoradiography. (B) Cells were preincubated with 50 μg/ml G. uralensisfor 30 min and followed by addition of 10 nM MSH for 1 h. Nuclear extracts were isolated and incubated with oligonucleotide probe AP-1. Transcriptional activation of AP-1 was assessed by EMSA and visualized by autoradiography.

Discussion

Traditional oriental herbal prescriptions have long been used for the treatment of several diseases in Oriental. This historical use of botanical products in the context of traditional medicine is being designated as 'alternative' or 'complementary' medical practice. Glycyrrhiza uralensis and GTG, one of the most commonly used traditional herbal medicines and prescriptions whose functions are as a tonic to the spleen, to moisten the lungs and the stop coughs, to relax spasms and stop pain, to moderate the action of herbs and to reduce fire and release toxins[2]. The major known ingredients of *G. uralensis* were determined by several methods such as TLC, HPLC, and capillary zone electrophoresis. These are includes glycyrrhizic acid, glycyrrhetinic acid, glycyrrhizin, uralenic acid, liquiritigenin, isoliquiritigenin, liquiritin, neoliquiritin, neoisoliquiritin, and licurazid[1]. The water extract of *G. uralensis* is using with combination of a mixture of crude extracts from other medicinal plants to develop and apply for new therapeutic agents. However, the mechanism study of melanogenesis protection by this agent was not investigated yet. It may involve modulation of the enzyme system responsible for intracellular signaling pathways including protein kinases such as cAMP-dependent protein kinase[25], protein kinase C-α[26], protein kinase C-β[27], and MAP kinase [28]. It may that melanogenesis is regulated by a network of several intracellular signaling mechanisms including the JNK pathway, but the molecular events connecting the regulation in tyrosinase activity also remain to be elucidated. This study was carried out to elucidate the protective mechanisms of GTGWE against α-MSH-mediated melanogenesis of mouse B16 melanoma cells.

Various pigmentary disorders and cosmetic applications require the use of depigmenting agents. Currently, available topical agents used for the reduction of pigmentation include tyrosinase inhibitors and melanocyte-cytotoxic agents[29]. In this paper, treatment of B16 melanoma cells with GTGWE resulted in a strong decreases of tyrosinase promoter activity and cell pigmentation (Fig. 2 and Fig. 4B). The amount of tyrosinase protein and the melanin contentwere correlated in both the transfected cells with tyrosinase promoter and the untransfected cells after treatment of GTGWE, suggesting the possibility that MAP kinase is involved in the regulation of melanogenesis through modulation of the proteinlevel of tyrosinase. Although MAP kinases have been clearly shown to play a crucial role in growth control, they are also involved in the differentiation process of several cell systems. MSH is also well known to target the B16 melanoma cells, stimulating melanization. Tyrosinase activation was also decreased after 72 h treatment of GTGWE in MSH-treated cells as well as untreated control cells, while it was enhanced upon MSH treatment (Fig. 4A). It might thus be speculated that the decreased expression of tyrosinase induced by GTGWE requires MAP kinase activation, and is thus inhibited by an elevation of cAMP, which reduces the extent of such activation. Consistent with this mechanism, Englaro and coworkers (1998) [10] recently reported that MAP kinase (ERK-2) pathway could be involved in the regulation of melanoma cell differentiation. Forskolin and cholera toxin stimulations are known to lead to an elevation of cAMP, which then activates PKA, MAP kinase, and AP-1, thus resulting in an increased transcription of tyrosinase[28]. Further subcutaneous injection of this hormone causes a strong stimulation of the local pigmentation in humans[30]. In this study, we showed that melanin synthesis, tyrosinase activity, and amount were simultaneously increased by addition of MSH. These observations confirmed previous reports suggesting that the control of tyrosinase mRNA expression is key step in the cAMP-mediated stimulation of melanogenesis in B16 melanoma cells.

AP-1 is a sequence-specific transcriptional activator composed of members of the Jun and Fos family[31]. Transcriptional activation of AP-1 is regulated by phosphorylation of c-Jun amino-terminal domain catalyzed by JNKs[24,32]. Many of the stimuli such as phorone, heat shock, inflammatory cytokines, and ultraviolet irradiation activate the JNK cascade, which is also called stress-activated protein kinase [33,34]. Recently, Mendelson et al. (1996)[35] showed that carbon tetrachloride stimulated JNK activation in the mouse liver. However, the mechanism by which stress culminates in JNK activation remains largely to be determined. Then Ap-1 could stimulate tyrosinase expression through the interaction with specific DNA sequences present in the tyrosinase promoter. To explore the upstream signaling pathways leading to tyrosinase gene suppression, we studied the involvement of JNK1 and AP-1. Interestingly, we found that GTGWE and α-MSH may act antagonistically on the regulation of gene expression as well as melanogenesis in B16 melanoma cells. Our result of time-point decrease in JNK activity after the administration of GTGWE is well coincided with the alterations of AP-1 binding activity (Fig. 5). We showed that a sustained decrease activation of the JNK1 pathway led to inhibit of AP-1 transactivation and consequently to a down-regulation of melanogenesis, demonstrating the involvement of this pathway in the control of B16 melanoma cell differentiation. Since some signaling mechanisms differ between melanoma cells and normal melanocytes, it is necessary to confirm that GTGWE

regulates melanogenesis by JNK pathway in normal melanocytes as in the melanoma cells.

Acknowledgement

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