Master's thesis

Effects on Photoaging and Wound Healing of Flavonoids

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February, 2015

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Effects on photoaging and wound healing of flavonoids

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A thesis submitted in partial fulfillment of the requirement for degree of Master of Science in Biomedicine and Drug Development

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This thesis has been examined and approved.

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CONTENTS

LIST OF ABBREVIATIONS

HDFs	Human Dermal Fibroblasts
Glycitin	4'hydroxy-6-methoxyisoflavone-7-D-glucoside
UV	Ultraviolet ray
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
RT-PCR	Reverse transcription polymerase chain reaction
MMP-1	Matrix metalloproteinase-1
ERK	Extracellular-signal-regulated kinases
JNK	c-Jun N-terminal kinases
P38	p38 mitogen-activvated protein kinases
MAPKs	Mitogen-activated protein kinases
SA-β-Gal	Senescence-associated β-galactosidase
SANA	n-succinyl-ala-ala-ala-p-nitroanilide
TDPN	2-(5-(2,4,6-trimethoxyphenyl)-4,5-dihydro-1H-pyrazol-3-yl)naphthalen-1-ol
EMT	Epithelial-mesenchymal transition
TGF-β1	Transforming growth factor beta 1
ECM	Extracellular matrix

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PART I

The Protective Effect of Glycitin on UV-induced Skin Photoaging in Human Dermal Fibroblast

1. ABSTRACT

Exposure of strong and repeated UV on the skin leads to skin aging, characterized with wrinkling, sagging, dyspigmentation, and laxity. Numerous studies revealed that Matrix metalloproteinases are related to skin aging and functions as degrading enzyme of various types of collagen. Here, we attempted to evaluate the effectiveness of glycitin (4'-hydroxy-6-methoxyisoflavone-7-D-glucoside) on skin aging and mechanisms of action in UV-irradiated human dermal fibroblasts. Especially we focused on the expression of Matrix metalloproteinase-1 (MMP-1), which degrades procollagen type-I in dermis, by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, Western blot, and reverse transcription polymerase chain reaction in cell lysates or media. Our results showed that glycitin increased the viability of human dermal fibroblast and alleviated MMP-1 expression caused by UV irradiation. In addition, synthesis of type-I collagen was increased and elastase inhibition activity was increased. UV-induced phosphorylation of ERK/JNK/p38 was decreased in dose-dependent manners. Taken together, we demonstrated that treatment with glycitin have a protective effect on skin aging by inhibiting of MMP-1 and increasing of collagen through ERK/JNK/P38 down-regulation, which may be mediated by the inhibition of ERK, JNK, and p38 mitogen-activated protein kinases. We suggest that glycitin is a potential agent for the treatment of skin ageing.

Key Word: 4'hydroxy-6-methoxyisoflavone-7-D-glucoside, collagen type-1, human dermal fibroblast, matrix metalloproteinase-1, skin ageing, UV

2. INTRODUCTION

Skin functions as a protective barrier between internal organs and the environment (Kalinin et al., 2002). Skin is divided into three regions: epidermis, dermis, and hypodermis. Dermis is the layer of skin beneath the epidermis that consists primarily of extracellular matrix proteins (Epstein and Munderloh, 1978). UV radiation is one of the causative factor that can affect the structure and functions of human skin (Bae et al., 2010). Chronic exposure to UV irradiation leads to skin aging which is characterized by wrinkling (Lee et al., 2008), sagging, dyspigmentation, and laxity (Kim et al., 2005).

Matrix metalloproteinases (MMPs) are a family of structurally related matrix-degrading enzymes that play important roles in photoageing as well as cell proliferation, migration, differentiation (Overall and Lopez-Otin, 2002), angiogenesis, and apoptosis (Werb, 1997). Among the MMP family, collagenolytic MMP (MMP-1) degrades fibrillar collagen in human skin (Chung et al., 2001).

Activity of MMPs and their regulation mechanisms have been investigated in UV-irradiated human dermal fibroblasts. UV irradiation induces the activation of protein kinase cascades, such as the mitogen-activated protein kinase (MAPK) cascade. A major effecter of the MAPK pathways is the transcription factor AP-1 (Whitmarsh and Davis, 1996). AP-1, which is composed of Jun and Fos family proteins (Janulis et al., 1999), induces MMP synthesis and inhibits collagen synthesis (Yu et al., 2013). Environmental stimuli, including UV irradiation, induces the transcription of Jun and Fos and subsequently they are activated by MAPKs; JNK, ERK, and P38 (Hibi et al., 1993).

Glycitin (4'-hydroxy-6-methoxyisoflavone-7-D-glucoside) is an isoform of glycitein, which is an O-methylated isoflavone, isolated from soy food products (Fig. 1). It has been reported to have an anti-oxidant and anti-carcinogenic ability (Chung Hung Lee et al.,

2005). However, the effect of glycitin on UV-induced skin ageing has not yet been investigated.

Thus, in the present study, the effects of glycitin isolated from soybean on MMP-1 and collagens in UV-irradiated human dermal fibroblasts were investigated. Moreover the molecular mechanism underlying the anti-photo ageing effects of glycitin was examined.

3. MATERIALS AND METHODS

3.1 Cell culture and UV irradiation

Human dermal fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 1% penicillin/streptomycin in a humidified incubator with 5% CO2 at 37oC. For all experiments, cells were cultured to 60% confluence and the medium was removed. The cells were washed with phosphate-buffered saline (PBS) and added fresh PBS. After washing, cells were irradiated with UV in PBS.

3.2 MTT assay

Cells were seeded on 96-well plates at a density of 3×104 cells/mL. Cells were irradiated with UV in PBS and then treated with glycitin or DMSO for 48 h. MTT solutions (10 μL of 5 mg/ mL solution; Amresco) was added to each well and incubated at 37oC for 4 h. Subsequently, the medium was gently removed and replaced with 150 μL of DMSO and incubated for 30 min with shaking to dissolve the precipitate. Absorbances of the samples were measured at 570 nm with a spectrophotometer (TECAN, Austria).

3.3 Western blot

Fibroblast-conditioned medium and cell lysates were collected, and the protein concentrations were determined with BCA protein assay reagents (Thermo Scientific, USA). Equal amounts of protein (30 μg/lane) were analyzed for each sample by resolving on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate gels were transferred to membranes. After being blocked in 5% non-fat dry milk with Tris-buffered saline Tween 20 (TBST) buffer, membranes were incubated with the indicated antibodies.

3.4 RT-PCR

Total cellular RNA was isolated using Trizol reagent (Invitrogen, USA). Subsequently, 2 μg of total RNA were used to synthesize cDNA using a Reverse Transcriptase Kit (Promega). The resulting cDNA was used for RT-PCR using the G-Taq kit (Cosmo Genetech, Korea) according to the manufacturer's instructions. The primer sequences were: forward 5'-GTCAGTTCCTTGAGCCG-3' and reverse 5'-GAAGGTAGAGCTTGGGCAGG-3' for MMP-1; forward 5'-GAGTCAACGGATTTGGTCGT-3' and reverse 5'-GACAAGCTTCCCGTTCTCAG-3' for β-actin; forward 5'-TGACGAGACCAAGAACTG-3' and reverse 5'-CCATCCAAA CCACTGAAACC-3' for human collagen type I. The results were analyzed using the ImageJ software.

3.5 Beta-galactosidase staining

UV irradiated and glycitin treated cells were fixed with 4% formaldehyde for 10 min. After washing with 'Washing solution (100mM of Na_2HPO_4, pH 6.0)', cells were incubated at 37℃ over night with 'staining solution (5mM of ferrocyanide, 5mM of ferricyanide, 2mM of $MgCl_2$, 150mM of NaCl, 40mM of Na_2HPO_4, 1mg/ml of X-Gal, pH 6.0)'.

3.6 Elastase inhibition activity assay

16μl of elastase (enzyme, 100μg/ml), 5~10 μM of glycitin, and 5.1 μl of SANA(n-succinyl-ala-ala-ala-p-nitroanilide, substrate, 50mM) were mixed and added Tris-HCl (pH 8.0, 200mM) up to 320μl. And then absorbance was measured at 410 nm using spectrophotometer (TECAN, Austria).

4. RESULTS

4.1 Inhibitory effects of glycitin on viability of UV-irradiated fibroblasts

To examine cell viability in UV-irradiated Human Dermal Fibroblasts (HDFs), the MTT analysis was performed. HDFs were cultured with various intensity of UV irradiation (0−80 mJ/cm^2) for 48 h, proliferation of irradiated cells were measured by MTT and proliferation was not affected (Fig. 2). According to MTT assay result, we selected intensity of UV at 80 mJ/cm^2 as the experiment condition. Furthermore, the effect of glycitin on proliferation was also determined. Treatments of glycitin on irradiated cells with 80 mJ/cm^2 at the concentrations of 5 and 10 μM for 48 h significantly increased cell viability by 10−20% as well as in non-irradiated cells (Fig. 3). These results suggest that UV irradiation does not have toxicity up to 80 mJ/cm^2, and glycitin increased cell proliferation in human dermal fibroblasts.

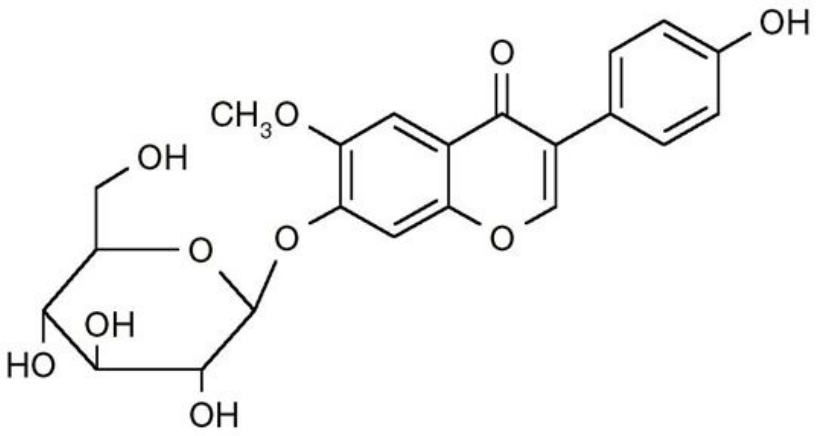

Fig. 1 The chemical structure of Glycitin

(4'-hydroxy-6-methoxyisoflavone-7-D-glucoside)

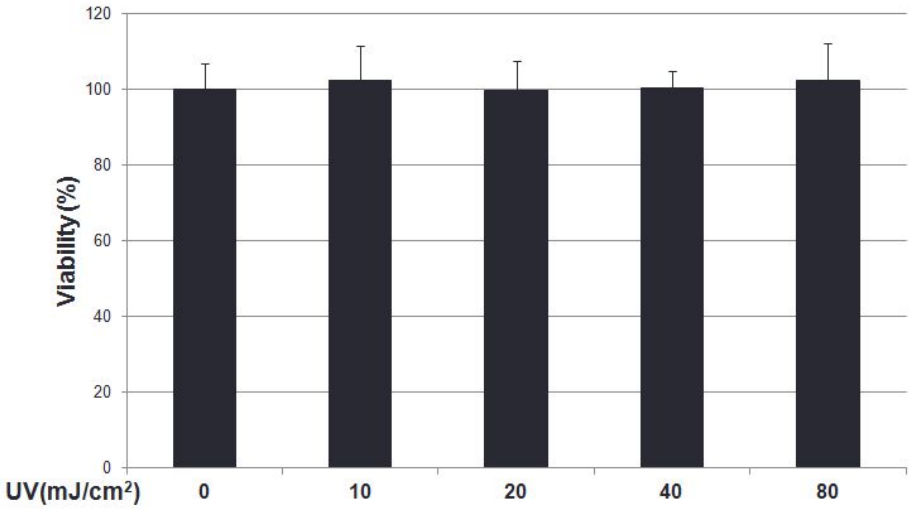

Fig. 2 UV irradiation did not affect proliferation of human dermal fibroblast

HDFs were irradiated with a different intensity (0-80mJ/cm^2), and proliferation was measured at 48h by MTT assay as described in Method. Data presented as mea of relative to control ±SD from 6 replication, (*) $p<0.05$.

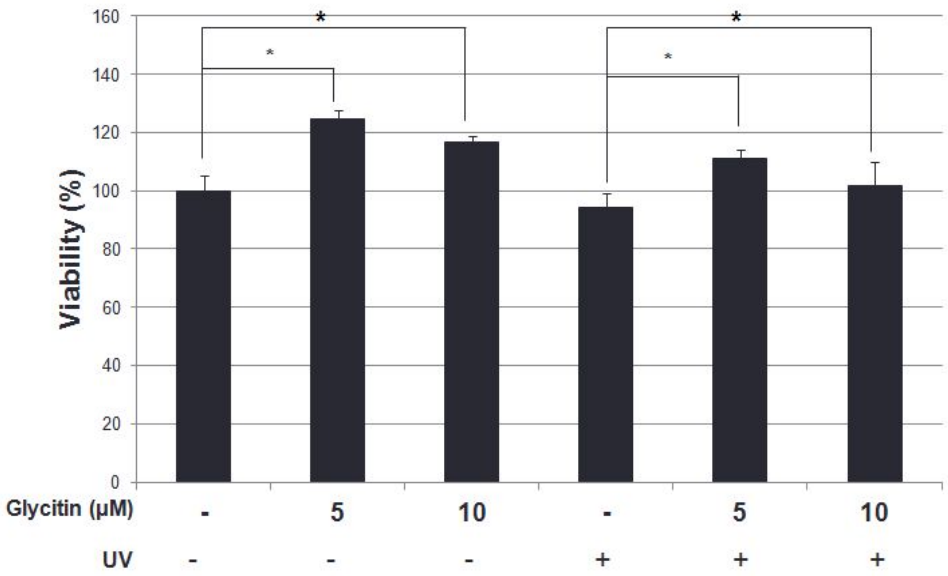

Fig. 3 Glycitin increases proliferation in human dermal fibroblasts

UV-irradiated HDFs were treated with 5 or 10 μM glycitin for 48h, and proliferation was measured by MTT assay. Data presented as mean of relative to control ±SD from 6 replication, (*) $p<0.05$.

4.2 Suppression of MMP-1 expression and secretion in UV irradiated and glycitin treated fibroblast.

UV irradiation is known to affect skin especially in dermis, which is the layer of skin beneath the epidermis that consists primarily of extracellular matrix proteins (ECM). UV irradiation degrades ECM caused by increased MMPs expression in UV-treated HDF. For this reason, the MMP-1 expression was observed by the Western blot. Human dermal fibroblasts were irradiated with UV at 0−80 mJ/cm^2, and then, cell lysates were analyzed by Western blot at 48 h after UV irradiation. The protein level of MMP-1 is increased in a dose dependent manner at 48 h after UV irradiation (Fig. 4). The expression of MMP-1 mRNA was measured using RT-RCR and also elevated by about 30% at 80 mJ/cm^2 compared to non-irradiated fibroblasts (Fig. 4). HDFs treated with 5 or 10 μM of glycitin for 48 h after UV irradiation at 80 mJ/cm^2. Fibroblast conditioned media and cell lysate were collected and measured the secretion of MMP-1 using Western blot. The secretion of MMP-1 was suppressed at least 70% in glycitin-treated cell lysates compared to control, but inhibitory effect of glycitin in media was distinguished from cell lysates by its concentration (Fig. 5). And the expression of MMP-1 mRNA was also suppressed out of glycitin at 10 μM and this result took on a similar aspect with cell lysates (Fig. 5).
These results suggest that glycitin inhibits the expression of MMP-1 protein and mRNA as well as secretion induced by UV irradiation in human dermal fibroblasts.

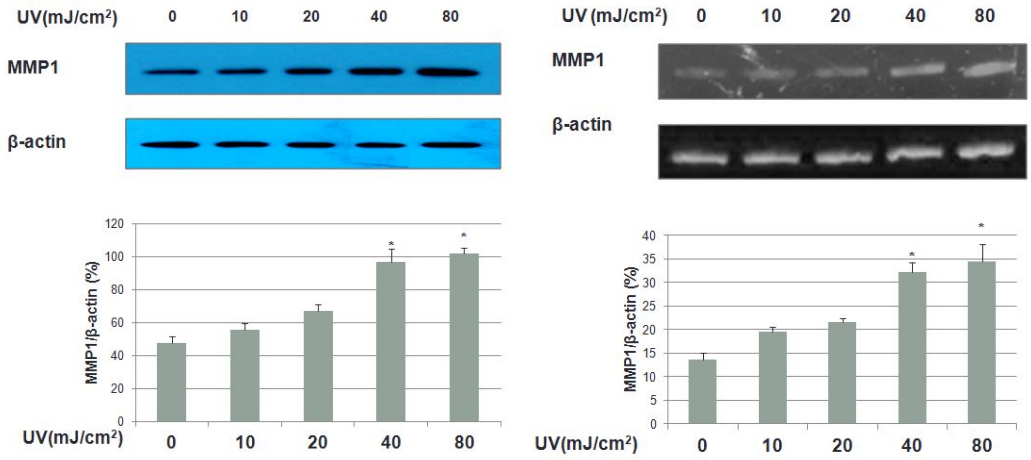

Fig. 4 UV irradiation increases MMP-1 expression in UV-treated human dermal fibroblasts

HDFs were irradiated with a different intensity of UV (0-80mJ/cm^2) for 48h, and cell lysates were immunoblotted with anti-MMP-1 antibodies. MMP-1 mRNA expression of HDF treated with UV and glycitin was evaluated by RT-PCR. Data presented as mean of relative to control ± SD from 3 replication, (*) $p<0.05$.

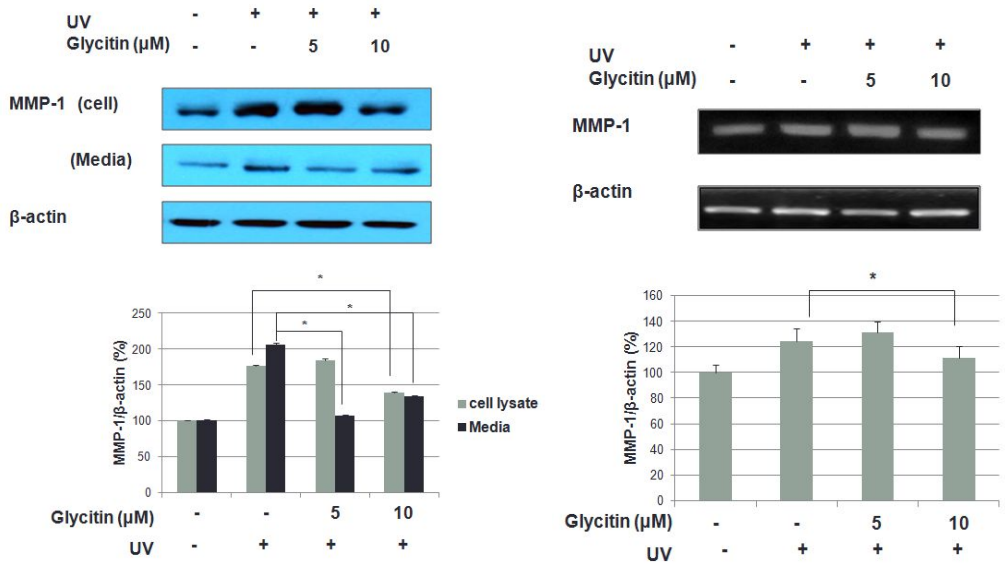

Fig. 5 Glycitin inhibits increased expression and secretion of MMP-1 induced by UV irradiation in human dermal fibroblasts

HDFs were treated with 5 or 10 μM glycitin after UV irradiation. Cell lysates and media were collected after 48h and measured MMP-1 expression and secretion by Western blot. MMP-1 mRNA expression of HDF treated with UV and glycitin was evaluated by RT-PCR. Data presented as mean of relative to control ± SD from 3 replication, (*) $p<0.05$.

4.3 Inhibition of collagen degradation in UV-irradiated fibroblasts by glycitin

MMP-1, a matrix-degrading enzyme, is well known to play important roles in photoaging and UV-induced MMP-1 in fibroblasts leading to the degradation of collagen I, III, and other extracellular matrix proteins. In particular, collagen type I is the most abundant protein in skin and activated by collagen type III, which also exists in dermis. However, aged skin features with reduction and impairment of the organization of collagen fibrils and elastin fibers. Therefore, in the present study whether UV irradiation degrades collagen was evaluated and whether glycitin attenuates the collagen degradation of dermal fibroblasts was induced by UV-irradiation. Cells were irradiated with UV (80 mJ/cm^2) and then treated with 5 or 10 μM glycitin. Fibroblast cell lysates and media were collected at 48 h, and Western blot was initiated. The results showed that glycitin increased collagen I expression and secretion by up to 66% in cell lysate and 81% in media (Fig. 6); the expression of collagen I mRNA was also increased in dose-dependent manner (Fig. 6).

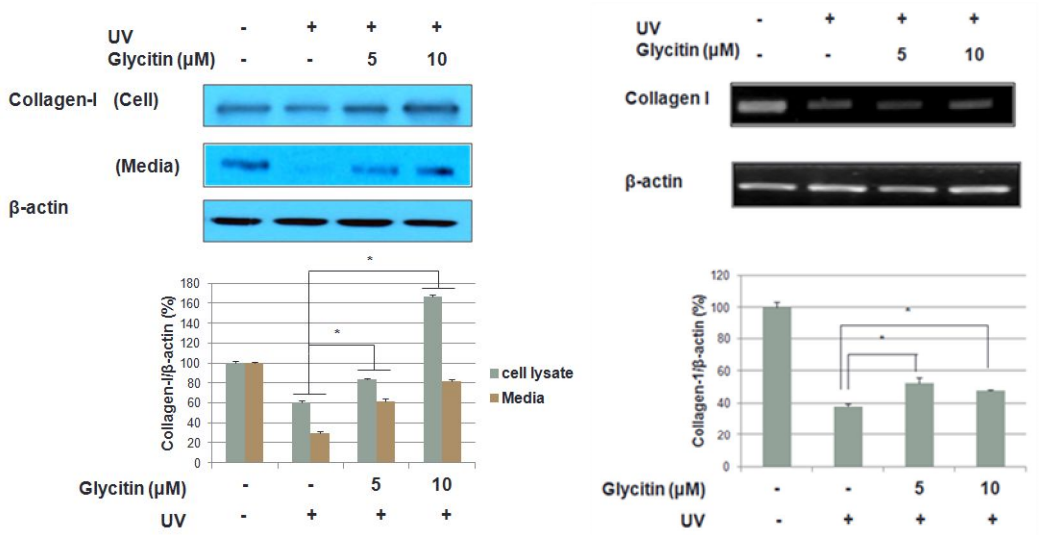

Fig. 6 Glycitin suppresses the degradation of collagen type I in UV-irradiated fibroblasts.

HDFs were treated with 5 or 10μM glycitin after UV irradiation. Cell lysates and media were collected at 48h and measured collagen type-I expression and secretion by Western blot. Collagen type-I mRNA expression of HDF treated with UV and glycitin was evaluated by RT-PCR. Data presented as mean of relative to control ± SD from 3 replication, (*) $p<0.05$.

4.4 Inhibitory effect of glycitin on elastase activity

Besides of collagenase 1 (MMP-1), elastase is also major cause of skin ageing. Decrease of elastin, which is an element of elastic fiber and provides flexibility in skin, by elastase leads to wrinkle formation in skin. Glycitin exhibited inhibitory effect on elastase activity in dose dependent manner (Fig. 7). Taken together, glycitin suppresses degradation of collagen in cell lysates and media or elastin by itself.

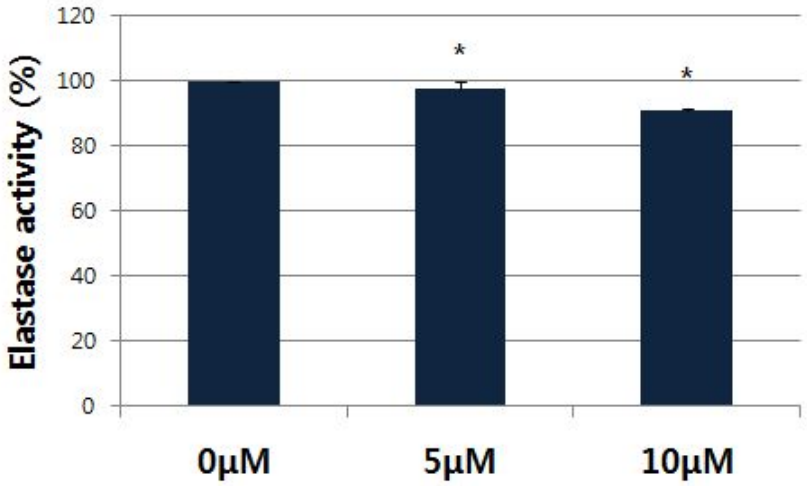

Fig. 7 Glycitin suppresses the degradation of elastin.

Elastase (enzyme), SANA (substrate), and glyctin were mixed with Tris-HCl (pH 8.0) and measured at 410 nm with a spectrophotometer. Data presented as mea of relative to control ±SD from 6 replication, (*) $p<0.05$.

4.5 Inhibitory effect of glycitin on activation of MAPKs signaling in UV-irradiated fibroblasts.

To examine the signaling of attenuation of glycitin related to UV irradiation, we focused on the MAPKs pathway that induces MMPs synthesis and inhibits collagen synthesis. Human dermal fibroblasts were irradiated with UV (80 mJ/cm^2), and treated with 5 or 10 μM glycitin. When cells were irradiated, phosphorylation of ERK, JNK, and p38 increased by at least two-fold compared to negative control (Fig. 7). This result suggests that UV irradiation induces MMP expression partly by activation of ERK/JNK/p38 pathway. However, treatment of glycitin on irradiated cells attenuated phosphorylation of ERK, JNK, and p38 in dose-dependent manner. Among them, p38 kinase was shown to be the most affected by glycitin (Fig. 7).

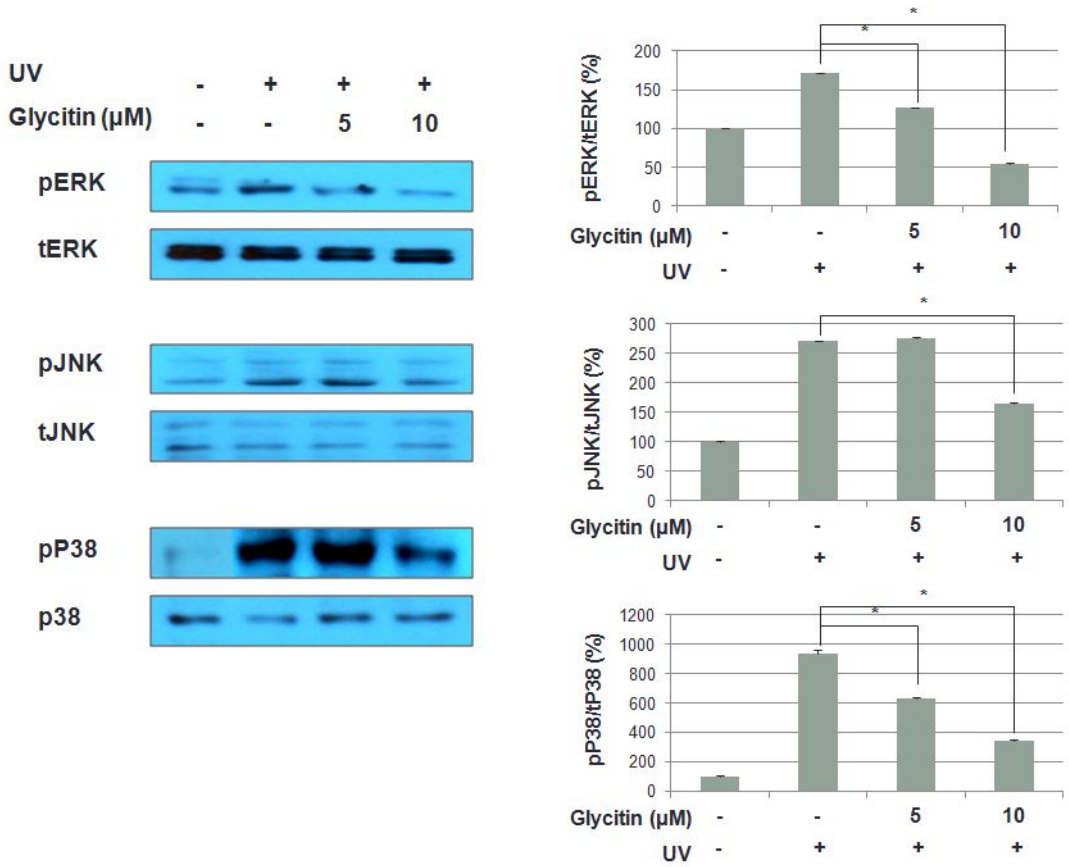

Fig. 8 Glycitin inhibits UV-induced MMP-1 expression by inhibiting MAPKs signaling.

UV-irradiated HDFs were treated with glycitin and cell lysates were collected. Expression and phosphorylation of ERK, JNK, and p38 were measured by Western blot using specific antibodies. Western blot results were quantified by ImageJ. Date presented as mean of relative to control ± SD from 3 replication, (*) $p<0.05$.

4.6 Inhibitory effect of glycitin on senescent Human Dermal Fibroblasts

To make sure of inhibitory effect of glycitin on senescent cells induced by UV irradiation, we stained with SA-β-Gal (Senescence associated-β-galactosidase). SA-β-Galactosidase is the enzyme that hydrolyzes β-Galactoside to monosaccharide in senescent cells, and it is dependent on pH change known as a feature of senescent cells. And X-gal is used for detecting expression of β-galactosidase in blue color by degrading. Human dermal fibroblasts were irradiated with UV at 80mJ/cm^2 and then treated with Glycitin. 2 days later, cells were stained with 'staining solution' and took a pictures using optical microscope (×200). When HDFs were irradiated with UV, beta-galactosidase expression increased as against control. But after UV irradiation glycitin-treated fibroblast were reduced in dose dependent manner (Fig. 8).

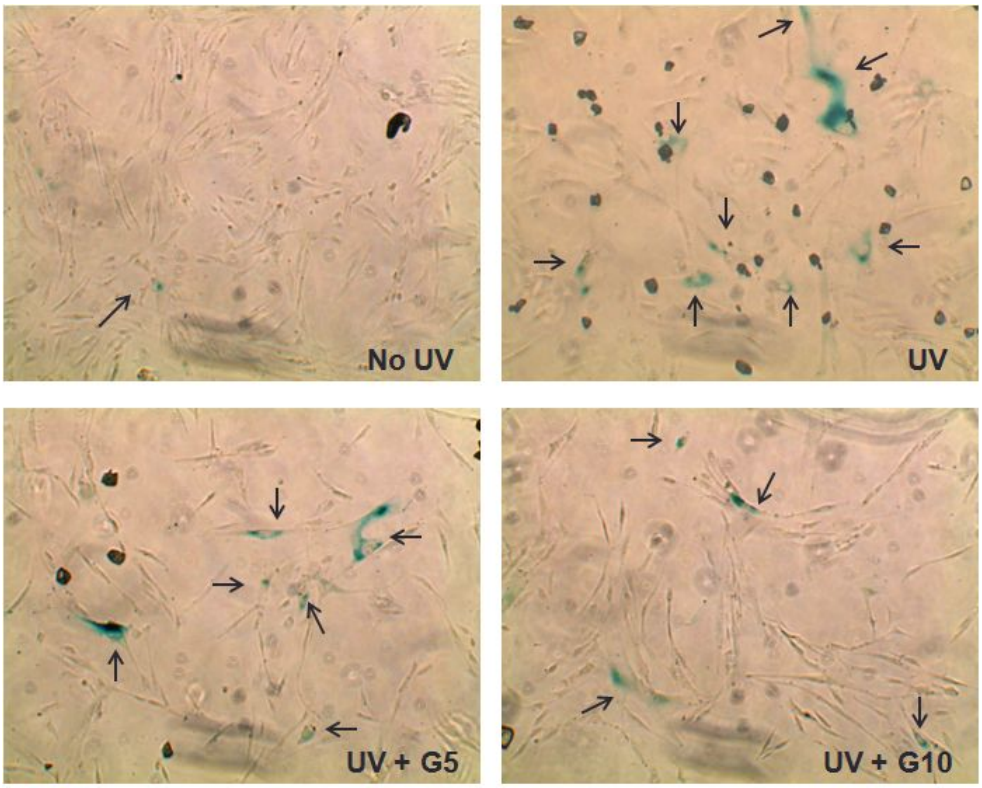

Fig. 9 Glycitin inhibits beta-galactosidase expression induced by UV irradiation in Human Dermal Fibroblasts.

UV-irradiated HDFs were treated with glycitin and stained with freshly prepared Senescence associated β-Glactosidase (SA-β-Gal) 48h later. (×200)

5. DISCUSSION

The present study demonstrates that glycitin may give inhibitory effects on UV-induced skin ageing through down-regulation of MMP-1. Glycitin is a flavonoid extracted from soy bean and is a glucoside form of glycitein (4',7-dihycroxy-6-methoxyisoflovone). Besides glycitin, soy bean extracts contain two more major isoflavonoids; daidzein (4',7-dihydroxyisoflavone) and genistein (4',5,7-trihydroxyisoflavone) and their respective beta-glycosides, genistin and daidzin (Hsieh et al., 2009). Glycitein, daidzein, and genistein function as ROS scavengers. These functions, which have been widely investigated, showed that they have different estrogenic activity by binding to estrogen receptor protein competitively (Song et al., 1999) and inhibit hydrogen peroxide induced cell damage by scavenging reactive oxygen species through inhibition of JNK pathway (Kang et al., 2007). However, the effect of glycitin on UV-induced skin ageing has not been investigated.

Treatment of glycitin attenuates UV-induced MMP-1 expression and secretion in human dermal fibroblasts (Fig. 6). MMP family plays a crucial role in the physiological mechanisms of skin ageing (Kim et al., 2013). In dermis, where the ECM is mainly located, activated MMP-1, which is activated by MMP-3, initiates degradation of type I and III collagens. Several approaches have been used to study new compounds and related pathways that inhibit the MMP expression resulting in ageing. For example, Carnosic acid (0−10 μM) attenuates UV-induced MMP-1, 3, and 9 expressions at 20 mJ/cm^2 in dose-dependent manner in human dermal fibroblasts (Park et al., 2013) and fucosterol also inhibits MMP-1, 2, and 9 expressions in UVB-induced HaCaT cells (Kim et al., 2013). In the present study, we found that glycitin inhibited UV-induced MMP-1 expressions in protein and mRNA level (Fig. 5). We assumed that a reduction of MMP-1 expression and secretion by glycitin are related to MAPKs signaling, leading to activation of AP-1. AP-1, which is composed of c-Jun and c-Fos family proteins, is known as an

important factor in synthesis of the MMP-1 and inhibits the collagen synthesis. Among the MAPKs, JNK binds to c-Jun and phosphorylates its N-terminal sites in response to UV irradiation (Minden et al., 1994) or binds to TCF (ternary complex factor) composed of c-fos along with p38 and ERK. Moreover, p38 phosphorylates ATF-2 (activation transcription factor 2) of c-Jun (Muthusamy and Piva, 2010), and further activated and stabilized c-fos and c-Jun form hetero-dimerization of AP-1, resulting in MMP-1 expression (Karin et al., 1997). Our results suggest that glycitin distinctly reduced MMP-1 expression through down-regulation of MAPK, which, in turn, led to deactivation of AP-1 complex at 10 μM of glycitin (Fig. 7).

As a result, reduction of MMP-1 leads to accumulation of collagen (Fig. 6). Collagens confer elasticity and strength to skin. Therefore, their degradation due to UV irradiation results in an aged features to the skin (Kim et al., 2013). The present study shows that UV irradiation augmented collagen degradation most likely via activated MMP-1 (collagenase-1) and disrupted collagen type-1 synthesis. However, glycitin significantly compounded the amount of collagen type-1 in media and also increased synthesis of collagen in a dose-dependent manner.

In conclusion, the present results demonstrate that UV irradiation might be a cause of skin photoageing through the decomposition of cellular matrix proteins such as collagen. However, treatment with glycitin showed a protective effect on skin ageing by inhibiting of MMP-1 and increasing of collagen through AP-1 down-regulation, which may be mediated by the inhibition of ERK, JNK, and p38 MAPKs. It was confirmed again using beta-galactosidase staining, biomarker of senescence. Therefore, we conclude that glycitin is a potential agent for the prevention and treatment of skin ageing.

PART II

A Novel Naphthochalcone Derivative Accelerates Dermal Wound Healing through Induction of Epithelial-Mesenchymal Transition of Keratinocyte

1. ABSTRACT

Wound healing is an intricate process whereby the skin repairs itself after injury. The epithelial-mesenchymal transition (EMT) is associated with wound healing and tissue regeneration. Naphthochalcone derivatives have various pharmaceutical properties. We investigated the effect of a novel naphthochalcone derivative, 2-(5-(2,4,6-trimethoxyphenyl)-4,5-dihydro-1H-pyrazol-3-yl)naphthalen-1-ol (TDPN), on dermal wound healing *in vivo* and the migration of keratinocytes *in vitro*. Furthermore, we investigated the effect of TDPN on signaling pathway and epithelial-mesenchymal transition through protein and transcriptional expression. The TDPN treatment accelerated dermal closure about 3 days and remodeling of dermis. We found that treatment with TDPN induced the migration of keratinocytes but not cytotoxicity. TDPN induced the phosphorylation of ERK and AKT. TDPN-treated cells showed loss of adherence protein and showed induction of the transcriptional factor Slug, mesenchymal marker, and fibronectin. Moreover, TDPN treatment induced the expression of matrix metalloproteinase-1 (MMP-1), which degrades specific components of the extracellular matrix, thereby providing new substrates that facilitate migration and invasion. MMP expression is considered to be one of the major attributes acquired by cells after EMT. We propose that a novel naphthochalcone derivative TDPN is capable of promoting keratinocyte migration *via* the induction of EMT resulting acceleration of wound closure and matrix remodeling.

Key Word : naphthochalcone · epithelial-mesenchymal transition · EMT · MMP · ECM

2. INTRODUCTION

Skin is the outermost organ of body that provides a barrier function to the body. In the epidermis layer of the skin, keratinocytes are the major cell population, constituting more than 90% of all the cells(Madison, 2003). When injuries to the skin occur, the keratinocytes at the wound site undergo morphologic alterations, changing from sedentary cells to migratory cells(Leopold et al., 2012). The phenotype of the migratory cells changes with respect to cell-cell adhesion and cell-matrix adhesion during the re-epithelialization stage. This phenotype partially resembles the epithelial to mesenchymal transition (EMT) process, which plays a critical role in cancer metastasis(Leopold et al., 2012). During both re-epithelialization and cancer metastasis, cells that undergo EMT lose contact with each other and the extracellular matrix (ECM)(Lauffenburger and Horwitz, 1996). The ECM may be degraded by matrix metalloproteinases (MMPs), which represent a family of zinc-containing endopeptidases. The relationship between the EMT and MMPs has been clarified. MMP expression is considered to be one of the major attributes acquired by epithelial cells after they undergo the EMT(Gilles et al., 2000). MMPs, such as MMP-1, MMP-2, MMP-9, and MMP-13, are frequently associated with processes that involve tissue re-modeling and cell migration. Membrane type 1-MMP (MT1-MMP) is spatially and temporally regulated during MCF10A cell migration owing to its mediation of pericellular proteolysis of the laminin-5 (Ln-5) g2 chain(Haas et al., 2003). The EMT is influenced by a wide range of regulatory factors. The signaling pathways for PI3K/AKT/mTOR and MAPK (ERK) are reported to be involved in the regulation of EMT(Steelman and Chappell, 2011, Leopold et al., 2012). Increased phosphorylation of AKT has been implicated in the induction of the EMT and cell migration(Irie et al., 2005). Furthermore, the transcriptional factors Snail and Slug play essential roles in cell migration. Slug expression has been shown to be up-regulated in the wound margins in the *in vitro*, *ex vivo*, and *in vivo* settings(Hudson et al., 2009). In Slug-

null mice, re-epithelialization is reduced, compared with wild-type mice(Hudson et al., 2009).

Pure chalcones, which are extracted from plants, display interesting biological properties, such as antioxidant, cytotoxic, anticancer, and antimicrobial activities(Batovska and Todorova, 2010). Naphthochalcone is a precursor to a variety of pharmaceutical agents. Naphthochalcone derivatives, which can be isolated from nature or biochemically synthesized, show various pharmaceutical effects (Zangade et al., 2011). One of the newly synthesized naphthochalcone derivatives, 1-(naphtho[2,1-*b*]furan-2-yl-carbonyl)-3,5-disubstituted-2,3-dihydro-1H-pyrazole, has been characterized by elemental analysis and in spectral studies. This compound has been evaluated for antimicrobial activity(Chandrashekhar et al., 2011). The compound (hydroxyphenyl) naphthol sulfonamide could be useful in optimizing 17β-HSD1 inhibitors for the treatment of endometriosis(Henn et al., 2012). Another derivative, of methyl-1-hydroxy-2-naphthoate, may inhibit lipopolysaccharide-induced inflammatory responses in macrophages *via* suppression of the NF-κB, JNK, and p38 MAPK pathways(Zhang et al., 2011). Regarding structural analogues of the flavones, alpha-naphtoflavone promotes pro-collagen production in skin fibroblasts and is suggested to have anti-aging effects(Liao et al., 2012). Recently we have screened series of compounds for promoting migration and proliferation of HaCaT cells and found that novel naphthochalcone derivative, 2-(5-(2,4,6-trimethoxyphenyl)-4,5-dihydro-1H-pyrazol-3-yl) naphthalen-1-ol (TDPN) (Fig. 1) had good activity.

The present study was aimed to examining whether novel naphthochalcone derivative (TDPN) exerts any effects on process of wound healing and, if so, what are the underlying mechanisms responsible for the action of TDPN. A murine excisional wound healing model *in vivo* and experiments using human keratinocyte cell line *in vitro* indicated TDPN accelerated wound closure by activating keratinocyte movement via

inducing EMT like change. The results of this study provide novel drug candidate of cutaneous skin wound.

3. MATERIALS AND METHODS

3.0 Reagent

The novel naphthochalcone derivative, 2-(5-(2,4,6-trimethoxyphenyl)-4,5-dihydro-1H-pyrazol-3-yl)naphthalen-1-ol (TDPN) (Fig. 1), was synthesized and kindly provided by Professor Youngho Lim (Division of Bioscience and Biotechnology, Konkuk University, Seoul, Korea). TDPN stock solutions were stored as aliquots at −20°C, and were diluted to the final concentration before use.

3.1 Cell culture

The human keratinocyte cell line HaCaT was cultured in RPMI medium that was supplemented with 10% fetal bovine serum (FBS; GIBCO) and 1% penicillin/streptomycin (PAA). Cells were incubated in a humidified atmosphere at 37°C in 5% CO_2.

3.2 Scratch wound healing assay

Because the doubling time of HaCaT cells is approximately 24 h, cells were seeded at 50% confluence in culture dishes 24 h before the wound healing scratch assay. Therefore, the experiment was performed when cells reached monolayer formation. A scratch was made in the cell monolayer by drawing a sterile p-200 pipette tip across the surface of the culture dish. After the scratch was made, the culture medium was supplemented with TDPN. Dimethyl sulfoxide (DMSO; Amresco) treatment was used as the control. At 0 h and 24 hours post-treatment, photographs of the cell monolayer were acquired at 4X magnification using the Olympus IX70 microscope equipped with a digital camera. The scratch was measured using the ImageJ software, and the difference between the initial and final width of the scratch was calculated.

3.3 MTT assay

Cells were seeded on 96-well plates at 200 µl of cells which have density of 3×10^4 cells/ml for each wells. Cells were treated with TDPN or DMSO for 24 h. MTT solution (10 µl of 5 mg/ml solution; Amresco) was added to each well and incubated for 37°C for 4 h. Subsequently, the medium was gently removed and replaced with 150 µl of DMSO and incubated for 30 min with shaking to dissolve the precipitate. The samples were measured at an absorbance of 570 nm in a spectrophotometer (TECAN, Austria).

3.4 Western blotting

Cells were treated with TDPN or DMSO in time-dependent and dose-dependent manners. The cells were harvested by scraping and underwent lysis in RIPA buffer. The BCA method (Thermo Scientific) was used for protein concentration determination. The extracts were analyzed by SDS-PAGE followed by Western blotting with appropriate antibodies.

The following antibodies were used for Western blotting: p21 (catalogue no. 2947), AKT (9272), phosphorylated AKT (9271S), ERK (4695), Slug (9585S), and GAPDH (2118) were from Cell Signaling Technology; E-cadherin (610181) and Zo-1 (610966) were from BD Science Transduction; cyclin E (sc-247), cyclin D1 (sc-246), p53 (sc-126), phosphorylated ERK (sc-7383), collagen I (sc-25974), collagen III (sc-28888), and fibronectin (sc-9068) were from Santa Cruz Biotechnology; and MMP-1 (444209) was from Calbiochem. The secondary antibodies used in the Western blotting were anti-mouse (PI-2000; Vector Laboratories) anti-rabbit (PI-1000; Vector Laboratories), and anti-goat (AP-107P; Millipore). The blots were analyzed using the ImageJ software. The relative change in the ratio of the target protein to the DMSO control was determined.

3.5 RT-PCR

Cells were seeded in a 60-mm culture dish and treated with TDPN. RNA from the treated

cells was extracted using the Trizol reagent (Invitrogen). Then, 2 μg of total RNA were used to synthesize cDNA synthesis using a Reverse Transcriptase Kit (Promega). The resulting cDNA was used for RT-PCR using the G-Taq kit (Cosmo Genetech, Seoul, Korea) according to the manufacturer's instructions.

RT-PCR was performed using the following gene-specific primers (forward and reverse primers, respectively), selected using the Blast Primer program: for *GAPDH*, 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGATTTC-3′; for *p53*, 5′-ACACGCTTCCCTGGATTGG-3 and 5′-CTGGCATTCTGGGAGCTTCA-3′; for *p21*, 5′-GTCAGTTCCTTGAGCCG-3 and 5′-GAAGGTAGAGCTTGGGCAGG-3′; for *MMP-1*, 5′-AGGGGAGATCATCGGGAC-3′ and 5′-GGCTGGACAGGATTTTGG-3′; for *MMP-2*, 5′- AACACCTTCTATGGTGCCC-3′ and 5′-ACGAGCAAAGGCATCACCA-3′; and for *MMP-7*, 5′-TACAGTGGGAACAGGCTAGG-3′ and 5′-GGCACTCCACATCTGGGC-3′.The results were analyzed using the ImageJ software. The relative change in the ratio of the target protein to the DMSO control was determined.

3.6 MMP zymography

MMP zymography was performed according to the method described by Gogly *et al.(Gogly et al., 1998)*, with the following modification: 8% sodium dodecyl sulfate (SDS) gels that contained gelatin (0.01 mg/ml) were used. The SDS in the gels was removed by incubating the gels twice (30 min each) in 200 ml of 2.5% Triton X-100 at 4°C. Thereafter, the gel slabs were incubated at 37°C overnight in the incubation buffer. The gels were then fixed and stained for 1 h with 0.05% Coomassie Blue R-250. The molecular mass protein markers were readily visible as stained bands against the lighter blue color of the stained gelatin background. Gelatinase activity was apparent as clear zones of lysis (negative staining) against the blue background. The gels were scanned to create a permanent record of the results.

3.7 Transwell invasion assay

The Transwell invasion assay was performed using a commercial Transwell plate (Corning). Cells were harvested by trypsinization and $7x10^4$ cells/well in 100 µl of medium were seeded into the wells of the insert. The receiving wells were set up with medium containing FBS as a chemoattractant. After 24 h, media in insert wells was replaced by 100ul of free serum medium containing TDPN, TGF-β1, or DMSO. Invasive cells in the receiving wells were collected by trypsinization and counted by hemacytometer.

3.8 Hoechst stain proliferation assay

The Hoeschst stain proliferation assay was performed as previously described (Richards et al., 1985). 200 µl of cells at a density of 3×10^4 cells/ml were seeded into the wells of 96-well plates. Cells were treated with TDPN or DMSO for 24 h. Hoechst 33342 solution (1 µl of 10 mM stock solution; Sigma) was added to each well and incubated for 37°C for 30 min. Cell proliferation was estimated by direct measurement of changes in cell fluorescence using a spectrofluorometer (SPECTRAFLUOR, Tecan). The instrument was equipped with a 365-nm broadband filter for the excitation beam and a 450-nm narrowband interference filter plus a UV blocking filter for the emitted light.

3.9 ECIS migration assay

HaCaT cells were seeded at a density of 1×10^5 cells/cm^2 into ECIS arrays and impedance was measured using an ECIS instrument. Wounding pulses of 2400 mA were applied for 20 seconds at 6400 kHz. Media was then replaced by media containing TDPN or TGFβ1 or DMSO. The results were exported to the ECIS program.

3.10 Trypan blue cell counting assay

$5x10^4$cells/well were seeded on 24-well plates. After 24 h of incubation, cells were treated

with TDPN or DMSO in various concentrations for 24 h. All cells in each well were collected by trypsinization. The live cells were identified and counted by trypan blue staining. This experiment was repeated three times.

3.11 Animal model

6-week old male ICR mice (n=5) were chosen for the experiment. The fur was removed with an electronic hair clipper and removal cream. Dermal wound was placed on the middle of a back using a 8mm punch instrument. 200 μl of TDPN was applied to the wounds of the experimental group in concentrations of 200 μM (dissolved with Vaseline) for 20 days, while the control group was treated with same amount of DMSO (dissolved with Vaseline). The wound was pictured every three day and the rate of wound closure was calculated as relative % of original wound area using Image J program. Skin samples were collected from mice at each of the wounding time points and fixed in 4 % formaldehyde.

These samples were used to stain with Hematoxylin/Eosin, Masson Trichrome and Ki-67 antibody.

4. RESULTS

4.1 TDPN induces the migration but not the proliferation of keratinocytes

Re-epithelialization involves the migration and proliferation of keratinocytes. In the present study, we investigated these two processes using the MTT assay and scratch wound healing assay for keratinocytes (HaCaT cells) treated with different concentrations of 2-(5-(2,4,6-trimethoxyphenyl)-4,5-dihydro-1H-pyrazol-3-yl)naphthalen-1-ol (TDPN) for 24 h. In the migration assay, TDNP promoted HaCaT cell migration dose-dependently up to 20 μM. However, there was little difference between 10 and 20 μM (Fig. 2A). It has been reported that TGF-β can induce EMT and migration of various cell types, including keratinocytes (Nicolas et al., 2003, Ellenrieder et al., 2001, Rasanen et al., 2010). To compare their abilities to induce migration, we performed migration assay comparing the ability of TGF and TDPN to induce migration, by both the ECIS system (Fig. 2B) and the Transwell invasion assay (Fig. 2C). These results demonstrated that TDPN induced migration and invasion comparably to TGF-β in the same time period of treatment.

Since migration rate may be influenced by proliferation rate, we tested cell growth. And keratinocyte proliferation was not affected by TDPN proven by three independent assays such as MTT assay, hemacytometer counting assay and in the proliferation Hoechst staining assay (Fig. 2D). These results also showed no significant changes between treated and untreated cells. To confirm the results, proteins related to the cell cycle were investigated in the treated cells. The levels of cyclin-dependent kinases E and D1, p53, and p21 were not significantly changed by TDPN (Fig. 2E). These data suggest that TDPN induces the migration but not the proliferation of keratinocytes.

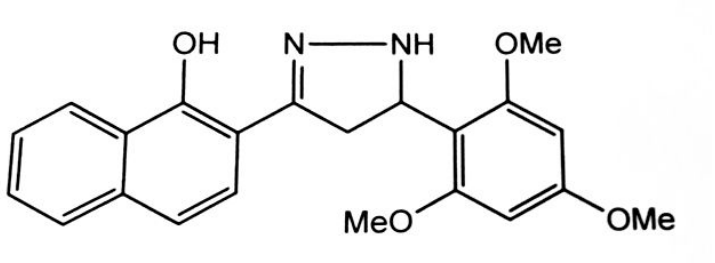

Fig. 1 Structure of TDPN

(5-(2,4,6-trimethoxyphenyl)-4,5-dihydro-1H-pyrazol-3-yl)naphthalen-1-ol

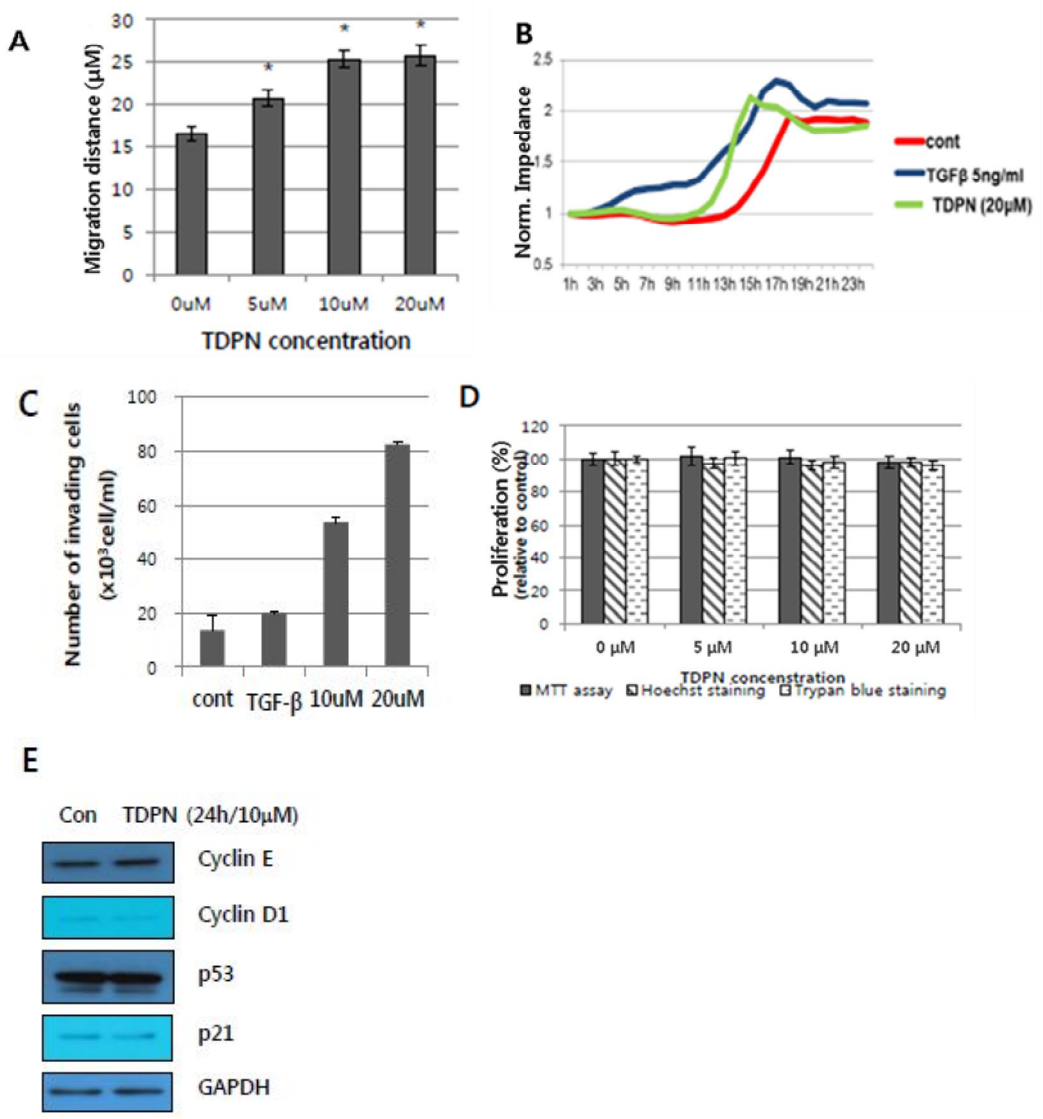

Fig. 2: TDPN effects on migration and proliferation of HaCaT cell line

Migration of HaCaT cells were measured by scratch wound assay and ECIS system (A-B). Invasion of TDPN treated cells were measured using transwell chambers as described in methods (C). Proliferation of keratinocyte cells were measures three different methods; MTT assay, Hoechst staining, and trypan blue counting assays (D). Western blot of cell cycle-related proteins comparing cells treated with 10 μM TDPN and untreated cells (E). (*)P<0.05 compare to control group.

4.2 ERK and AKT phosphorylation is involved in promotion of keratinocyte migration by TDPN

It is well-known that mitogen-activated protein (MAP) kinase family members, ERKs, as well as the PI3K/AKT pathway, are important for the regulation of cell migration. Therefore, we investigated the expression levels of regulatory proteins related to cell migration, such as AKT and ERK. Keratinocytes were treated with TDPN for different time periods and protein lysates were assayed for phosphorylation of AKT and ERK. We used a stock of TDPN containing approximately 0.05% DMSO. Each time point, cells treated either DMSO or TDPN in DMSO were collected and assayed by western blot analysis. The relative intensity between TDPN and control group at each specific time point was calculated (Fig. 3A). Within 2h ERK and AKT signals were activated. These signals were increased dose-dependently at 2h time point (Fig. 3B) and the treatment of specific inhibitors against ERK and AKT attenuate TDPN induced phosphorylation (Fig. 3C). To identify the TDPN induced migration of keratinocyte was mediated by ERK and AKT signaling, we performed a wound healing assay in which cells were pretreated with a PI3K inhibitor (LY49002, 50 μM) and a MEK inhibitor (PD98059, 50 μM) then treated with TDPN or left untreated. The results demonstrated that inhibiting the AKT and ERK pathways abolished the effects of TDPN on migration (Fig. 3D).

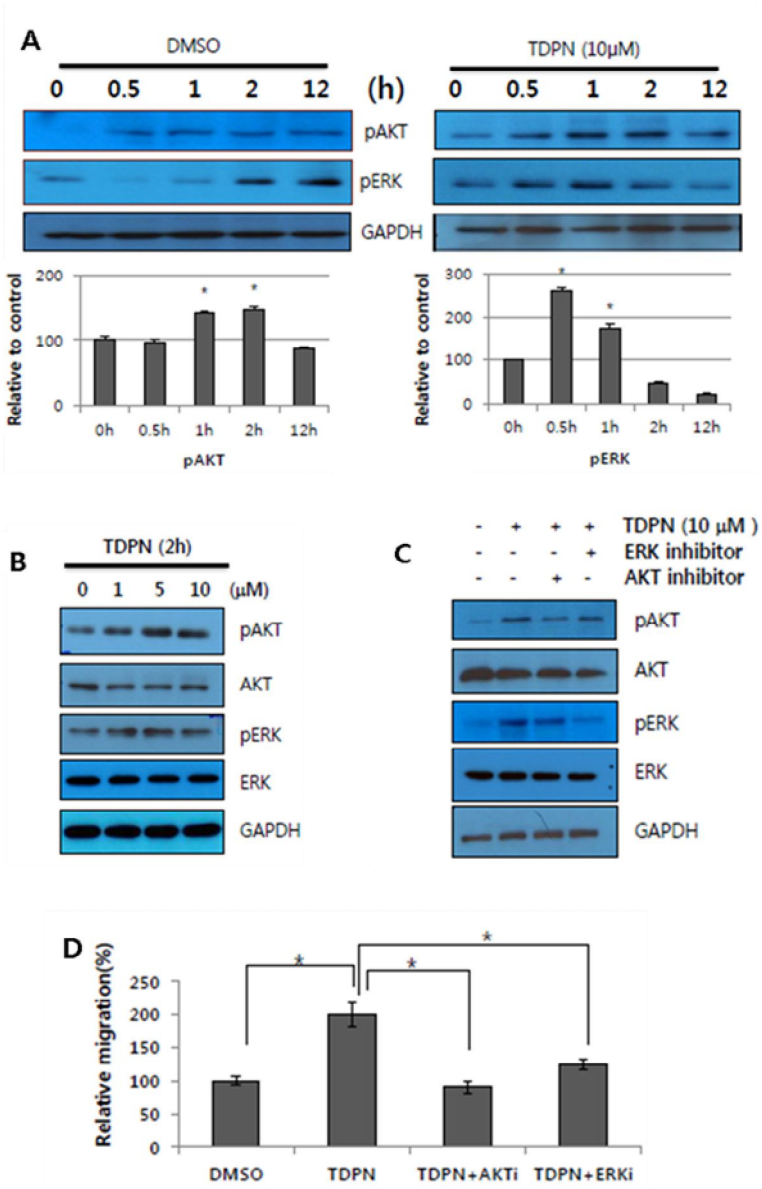

Fig. 3: ERK and AKT phosphorylation is involved in promotion of keratinocyte migration by TDPN

HaCaT cells were treated with either DMSO or 10 μM TDPN for the indicated time (A) or the indicated dose for 2 h (B) and harvested for western blot analysis. Cells were pre-treated with PI3K inhibitor (LY49002, 50 μM,) or MEK inhibitor (pd98059, 50 μM,) for 24h, then treated with TDPN for 1 h and harvested for western blot analysis (C). After co-treatment of TDPN and PI3K inhibitor (LY49002, 50 μM) or MEK inhibitor (pd98059, 50 μM) for 24hrs, scratch wound healing assay were performed. Distance of the scratch was measured relative to control after 24 hours of treatment with TDPN (D). (*)P<0.05)

4.3 TDPN induces EMT like change

It has been reported that EMT occurs in keratinocytes at wound sites during the re-epithelialization stage (Kalluri and Weinberg, 2009, Thiery et al., 2009). Thus, we investigated whether TDPN promotes re-epithelialization through EMT. We treated TGF-β1 (10ng/ml) and TDPN for 24 h and observed morphological and biochemical changes. On above condition, morphological changes were not dramatic (S1). It has been reported that TGF-β induces invasion and changes the cell size of several cell types including epithelial cells and keratinocytes (Nicolas et al., 2003, Ellenrieder et al., 2001, Rasanen et al., 2010). However, morphological change of keratinocytes by TGF-β on *in vitro* varies depending on cell types. HaCaT cells treated with TGF-β1 for 24 h only form actin stress fibers but morphology does not change (Brown et al., 2004). However, in cellular level, main EMT markers such as epithelial marker proteins Zo-1 and E-cadherin, and mesenchymal marker, transcriptional factor Slug changed (Fig. 4A). Western blot results revealed that TDPN treatment increased Slug expression and decreased the E-cadherin and Zo-1 in a time-dependent manner (Fig. 4A) and dose-dependent manner (Fig. 4B). This suggests that TDPN treatment causes the loss of cell-cell adhesion. In addition, the level of the mesenchymal marker fibronectin was slightly increased (Fig. 4D).

In both EMT tumors and migrating keratinocytes, degradation and remodeling of the ECM are needed. The EMT pathway has been reported to involve MMP expression(Gilles et al., 2000). In the present study, zymography revealed that the levels of MMP-2 and MMP-9 were not changed by TDPN treatment (S2). However, the Western blot results for TDPN-treated cells showed that the MMP-1 protein level was significantly increased (Fig. 4C).The transcriptional expression pattern showed a similar result (Fig. 4D).

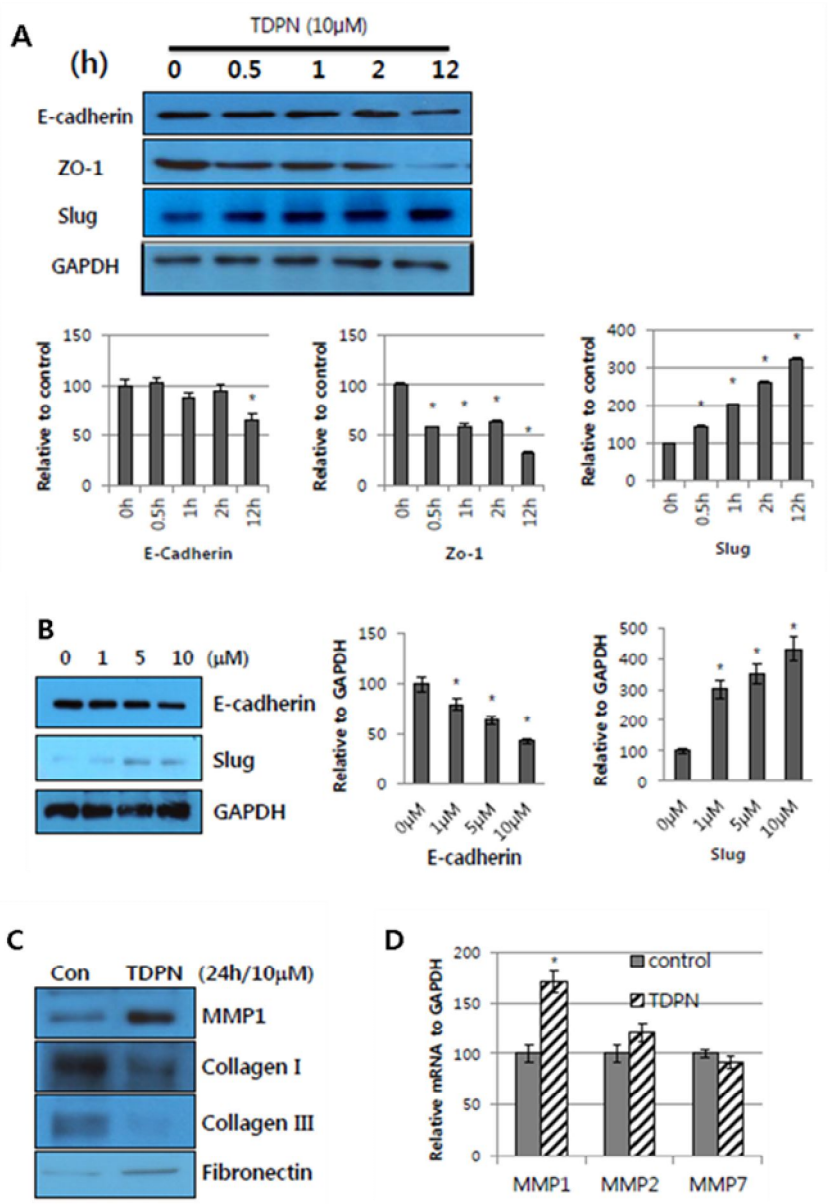

Fig. 4: TDPN induces EMT like change

HaCaT cells were treated with TDPN (10 μM) for the indicated time (A) or at the indicated dose (B) and harvested for western blot analysis. HaCaT cells treated with 10 μM TDPN for 24 h. After treatment, tProtein in media was collected and analysed by western blot (C). Transcriptional expression of MMPs in HaCaT cells treated with TDPN (10μM for 24 h). After treatment, total RNA from each cell was taken by Trizol reagent and analysed by RT-PCR (D). (Data presented as mean of relative to GAPDH ± SE from least 3 replications, (*)P<0.05 compare to control group)

4.4 TDPN accelerated dermal wound healing

Full-thickness excisional wound were made on the dorsal of mice and TDPN or Vaseline were applied to wound site topically every day. TDPN treated mice exhibited a faster wound closure then muck treated animals as early as day 9 (Fig. 5A). The average of opened wound area were measured by image program and the rate of closure of TDPN treated was 3day faster than untreated ones (Fig. 5B). Tissue samples were taken at day 9, 12 and 15 and performed H&E staining. On day 9, TDPN treated wound revealed that epidermal closure is complete but dermal closure is not sufficient to have open area (yellow arrow) and inflammation and necrosis is still remain in outer skin (Fig. 6A). Whereas muck treated wound showed that necrosis and inflammation accompanied in wound site and epidermal leading edge is observed (black arrow) but epidermal closure is not complete. The granulation tissue formation in dermis is incomplete and dermal closure is ongoing (Fig. 6B). On day 12, in TDPN treated wound, epidermal and dermal closure is complete and hair follicle start to show but not yet start differentiation. Contraction of granulation tissue squeezes hair follicle to wound direction (Fig. 6C). In muck treated wound, granulation tissue formation is processed and dermal closure is complete, however, the proliferation of epidermis is not sufficient and the epidermal closure is incomplete (Fig. 6D). On day 15, in TDPN wound, epidermal and dermal closure is complete perfectly and glands differentiation is processing throughout the wound site (Fig. 6E). Day 15 muck treated wound still have no sign of a nodule that resembled early sweat or sebaceous glands (Fig. 6F). Collagen deposits were stained by Masson Trichrome (Fig. 6G-J). In TDPN treated animal, until day 12 collagen deposition is still ongoing and distribution of collagen fiber is uneven (Fig. 6G). In muck treated wound, the collagen deposit did not show much difference compared to TDPN treated (Fig. 6I-J). Results suggested accelerated wound closure may attributed by faster epidermis differentiation.

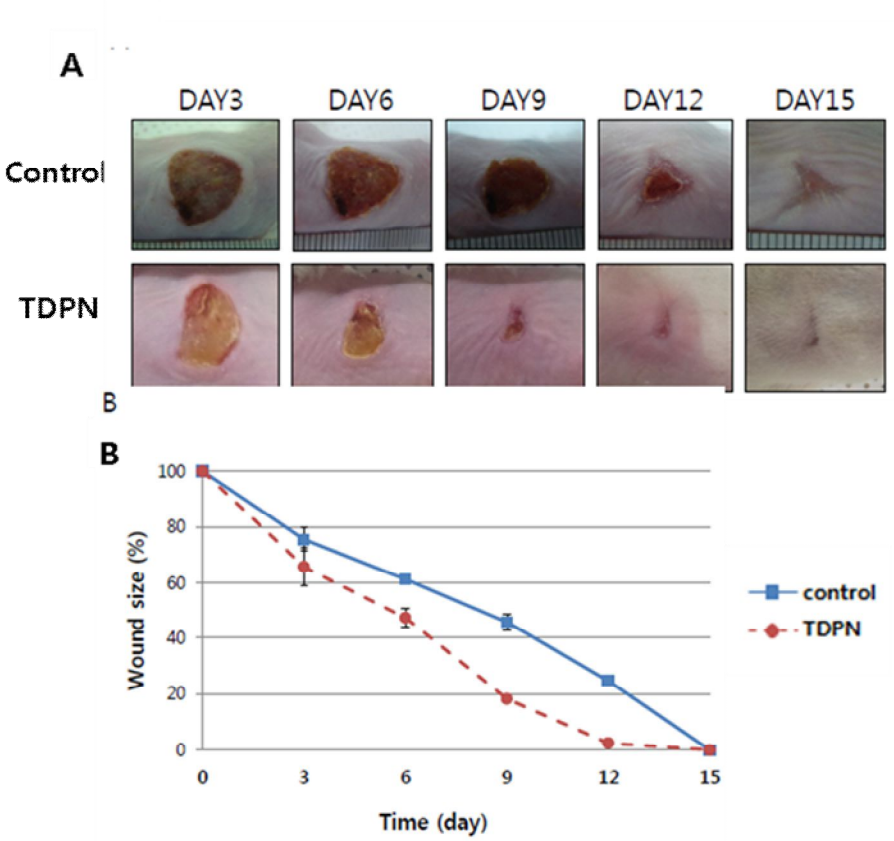

Figure 5: Wound closure after a full-thickness dermal excision.

Male ICR mice (6 weeks of age) underwent a full-thickness 8 mm excisional wounding. They were either muck treated or treated with cutaneous application of TDPN (200 μM) daily. The wound closure was pictured every three day (A). The averages of opened wound area were measured by image program and plotted as relative % of original wound (B). The values were expressed as means ± SE from 6 wounds in each group till day 9, 4 wounds at day 12 and 2 wounds at day 15.

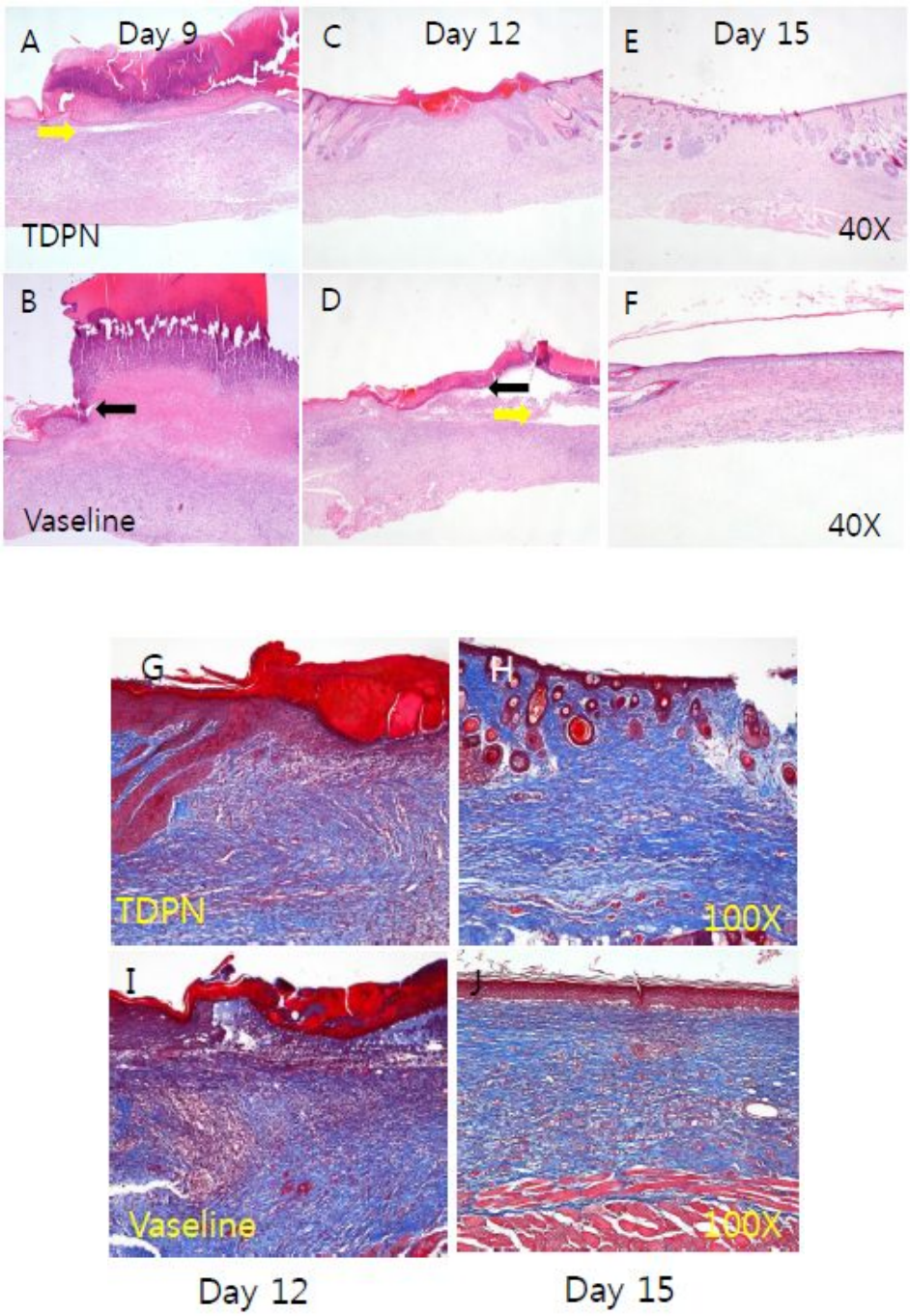

Figure 6: Histological findings of dermal wound tissues during the healing process.

Dermal specimens were obtained from ICR mice either TDPN treated (A, C and E) or muck treated (B, D, and F) as in Fig. 5, at day 9(A and B), day 12 (C and D) or day 15(E and F). They were subjected to H-E staining (A-F) or Masson Trichrome staining (G-J). Yellow arrow indicated open area. Black arrow indicated epidermal leading edge. Represented pictures are shown from 2 wounds in each group at each day.

4.5 TDPN promote epidermis development via keratinocyte activation

We performed Ki-67 antibody staining for detect proliferation (Fig. 7). In both case, the day 9 show highest proliferation index (Fig. 7A-F). In the TDPN group at day 12 and muck treated group at day15, cells located at the bilayer of epidermis (yellow arrow) show strong reaction as seen in normal epidermis (Fig. 7B, F). However, on day 15, TDPN treated group show high proliferation not only epidermal bilayer but also around nodules that resembled early sweat or sebaceous glands (Fig. 7C). These results suggested that TDPN mainly activated epidermal keratinocyte rather than dermal fibroblast.

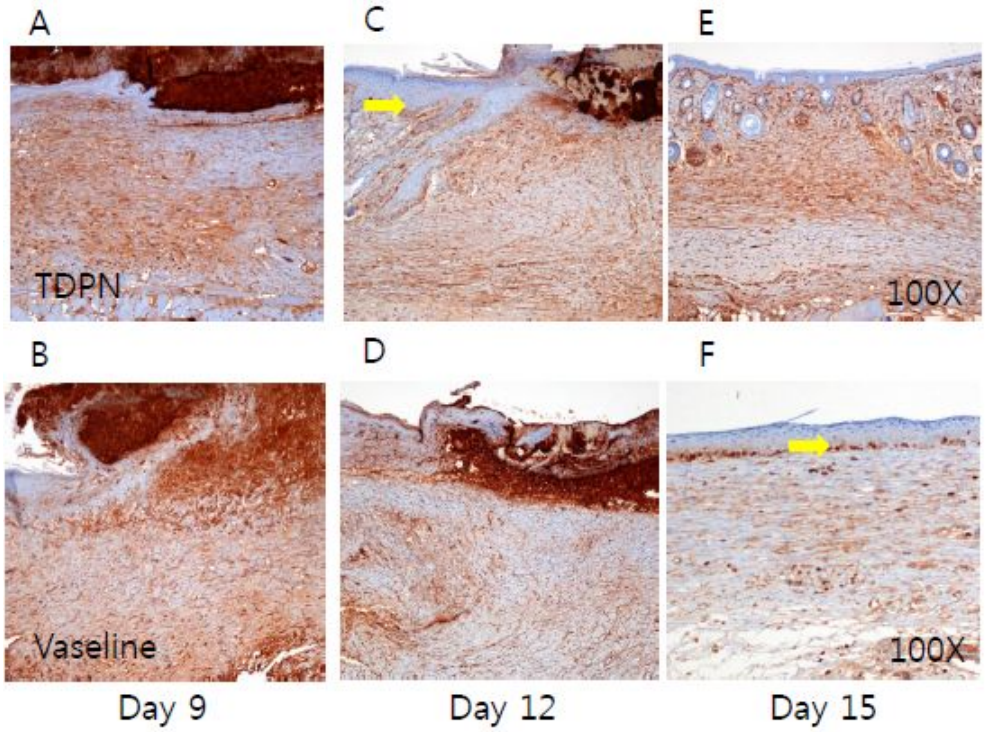

Figure 7: Proliferation of dermal wound tissues during the healing process.

Dermal specimens were obtained from ICR mice either TDPN treated (A, C and E) or muck treated (B, D, and F) as in Fig. 5, at day 9(A and B), day 12 (C and D) or day 15(E and F). Proliferation of cell in wound site was measured by ki-67 antibody staining. Represented pictures are shown from 2 wounds in each group at each day.

5. DISCUSSION

Our results reveal that the novel naphthochalcone derivative TDPN show faster wound closure and matrix remodeling (Fig.5-6) via promoting the migration but not the proliferation of keratinocytes (Fig.2). Importantly, we observed promotion of the EMT pathway in the TDPN-treated cells. The expression levels of the adherence junction protein E-cadherin and tight junction protein ZO-1 were reduced in the cells treated with TDPN. Also, the expression levels of the transcriptional factor Slug, as well as those of the mesenchymal marker fibronectin were induced in these cells (Fig. 4). Furthermore, the ERK and AKT signaling pathways were found to be involved in TDPN induction of EMT, as evidenced by the induction of phosphorylation of ERK and AKT which was confirmed by pretreatment AKT inhibitor (LY49002, 50 μM) or ERK inhibitor (PD98059, 50μM) attenuated effect of TDPN (Fig. 3). As a consequence of the EMT, MMP-1 (but not MMP-2 or MMP-9) was found to be significantly induced. Following the induction of MMP-1 in the TDPN-treated cells, the ECM proteins collagen I and collagen III were significantly degraded.

The TDPN mainly stimulate keratinocyte not fibroblast. Keratinocyte migration, a part of cell proliferation stage, plays an important role during the wound healing(Reinke and Sorg, 2012). Several approaches have been used to study new compounds and new pathways that promote migration and wound healing(Yang et al., 2010, Park et al., 2011). For example, Protection effects of green tea extract (EGCG) against UV were found to be involved in the proliferation of normal human keratinocytes (Chung et al., 2003). EGCG-induced Erk phosphorylation and activation of the Akt pathway was found to promote keratinocyte survival. However, in our study, TDPN showed effects on the migration but not on the proliferation of keratinocytes. VPA cutaneous wound healing by increasing the motility of HaCaT keratinocytes through ERK and phosphatidylinositol 3-kinase (PI3-kinase)/Akt signaling pathways(Aliouat-Denis et al., 2005).

The epithelial-mesenchymal transition (EMT) is one of the major factors that affect cell migration associated with wound healing and tissue regeneration(Kalluri and Weinberg, 2009, Thiery et al., 2009). Loss of cell junctions is considered to be a crucial marker for EMT(Thiery et al., 2009). Extensive previous studies have focused on how tight junctions are down-regulated in EMT(Haas et al., 2003, Ikenouchi et al., 2003, Acloque et al., 2009)_ENREF_25. In addition, during the early stage of re-epithelialization, when cells undergo EMT, reduced cell-cell contacts allow the cells to migrate. EMT down-regulation of the cell junction protein E-cadherin appears to involve regulation by Wnt or TGF-β *via* inducible activation of kinase pathways that modulate GTPase, Smads, PI3Ks, MAP kinases, β-catenin, and activate transcription factors, including LEF-1, Snail, Slug, and Scatter, ultimately leading to repression of the E-cadherin gene(Kalluri and Neilson, 2003). Apart from the loss of cell junctions, the increased expression levels of Slug and mesenchymal marker act as a marker for EMT(Bolós et al., 2002, Kalluri and Weinberg, 2009). Slug, a key transcription factor is responsible for the down-regulation of E-cadherin in both explanted human skin and primary keratinocytes(Yan et al., 2010).

The cell migration is regulated by various signaling pathways. The MAPK signaling pathways are associated with cell migration(Huang et al., 2004), as well as the EMT pathway(Shi et al., 2008, Shin et al., 2010), and the PI3K/AKT signaling pathway has also been implicated in cell migration(Kandel et al., 2009, Steelman and Chappell, 2011) and EMT. TDPN induced phosphorylation of ERK and AKT in time- and dose-dependent manners (Fig. 3). A previous report showed that transforming acidic coiled-coil protein 3 (TACC3) induced EMT and migration through induction of the AKT and ERK pathways(Ha et al., 2013). Furthermore, it has been reported that inhibition of the ERK and AKT pathways leads to inhibition of the induction of EMT by TGF-β1(Davies et al., 2005). Our results revealed that the phosphorylation of signaling pathway proteins AKT and ERK were induced early in TDPN treatment, while changes of EMT markers such as

Slug and E-cadherin detected later. We suggest that TDPN triggers early signals in the EMT pathway (Fig. 3-4).

Matrix metalloproteinases (MMPs), which are extracellular proteases that are highly expressed at wound sites, degrade specific components of the ECM, thereby providing new substrates that facilitate migration and invasion(Stevens and Page-McCaw, 2011). The relationships between EMT and MMPs have been described. MMP expression is considered to be one of the major attributes acquired by epithelial cells after EMT(Gilles et al., 2000). MMP-2 and MMP-9 have been reported to play essential roles in the EMT in the avian embryo(Duong and Erickson, 2003, Lin et al., 2011). Interestingly, our enzymatic activity results do not accord with the results in the previous report, in that the levels of MMP-2 and MMP-9 were not changed in our treated cells. However, MMP-1 (type I collagenase) is needed in the epidermis for re-epithelialization(Stevens and Page-McCaw, 2011). In human oral keratinocytes, MMP-1 mRNA was expressed while the keratinocytes covered the wound surface(Garlick et al., 1996). In agreement with those results, MMP-1 was induced in the TDPN-treated cells in our study (Fig. 4). *In vitro* keratinocyte culture, collagen I and collagen III were significantly degraded in the TDPN treatment group. Given that MMP-1 is a collagenase, its induction would lead to the degradation of the collagen helping keratinocyte migration at the margin of dermis.

The finding of novel TNPN to promote wound healing via keratinocyte migration supply good tool for studying wound healing mechanism and possible drug candidate.

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Ⅳ. 요약문

I. Glycitin의 광노화 억제 효과

사람의 피부가 강한 UV에 지속적으로 노출되면 피부노화가 진행되어 주름, 색소침착, 처짐 등의 특징을 나타낸다. 또한 많은 연구들을 통해 세포 외 기질 분해효소(MMP)가 세포 외 기질을 분해함으로써 노화를 일으킨다는 것을 밝혀냈다. 따라서 우리는 UV가 조사 된 human dermal fibroblast를 이용하여 glycitin이 피부노화에 어떤 효과를 나타내는지 알아보고자 하였다. 특히 콜라겐-1을 분해하는 MMP-1 효소의 발현에 초점을 맞춰 MTT assay, Western blot과 reverse transcription polymerase chain reaction 등의 실험을 진행하였다. Glycitin은 UV가 조사 된 human dermal fibroblast의 증식을 증가시켰고, MMP-1의 발현을 감소시켰다. 또한, 콜라겐-1의 합성과 elastase의 억제율을 증가시켰고, UV에 의해 유도 된 ERK/JNK/P38의 인산화를 농도 의존적으로 억제하는 결과를 나타냈다. 이를 종합하여, 우리는 glycitin이 ERK/JNK/P38 경로를 통해 MMP-1과 elastase의 작용을 억제함으로써 피부 노화를 보호할 수 있는 잠재적인 물질이라고 기대해 볼 수 있었다.

II. 새로운 Naphthochalcone 유래물질의 상피-간엽세포 전환을 통한 상처 치유 효과

상처 치유는 피부가 손상되었을 때 재생하는 과정을 뜻한다. 상피-간엽세포 전환(EMT)은 상처 치유와 조직 재생과 관련이 깊다. Naphthochalcone 유래물질은 다양한 치유의 기능을 가진다. 우리는 새로운 naphthochalcone 유래물질인 TDPN이 진피 층의 상처 치유와 (in vivo) 각질세포의 이동성 (in vitro)에 미치는 영향을 확인해보았다. 또한, 단백질 발현과 전사 과정에서 TDPN의 EMT 경로를 확인하였다. TDPN을 처리했을 때, 3 일째부터 진피 층의 재생이 빠르게 진행되었다. 우리는 TDPN이 각질세포에서의 세포독성이 아닌 이동성을 증가시키는 것을 알아냈고, 이는 ERK와 AKT의 인산화를 통해 일어난다는 것을 확인하였다. TDPN을 처리한 각질세포에서 접착 단백질의 발현 감소와 Slug와 Fibronection이 유도되었다. 게다가 이동성과 침투성을 유도하는 MMP-1의 발현이 증가하였다. 이를 통해 우리는 TDPN이 EMT 경로를 통해 상처 치유와 기질의 재생을 일으킴으로써 각질세포의 이동성을 증가시키는 것을 확인하였다.

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먼저 **조문제 교수님**께 감사의 인사를 드리고 싶습니다. 백지상태였던 저에게 배울 수 있는 기회를 주셨어요. 당장 일어날 일 보다 미래를 내다볼 수 있는 시야를 갖도록 많은 조언도 해주셨습니다. 또 저희를 위해 아낌없는 지원해주시느라 동분서주하시는 것 알지만 감사인사 한 번 드리지 못했어요. 감사합니다 교수님. 앞으로 제자리걸음이 아닌 발전하는 모습 보여드리도록 노력하겠습니다. 항상 건강하시고 지금처럼 멋진 모습으로 남아주시길 바랍니다.

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학부, 석사 생활 통틀어서 첫 후배가 생겼습니다. **Sophors**에게도 고맙다고 전해주고 싶습니다. 많이 미흡한 선배이지만 열심히 배우려고 하고 궂은일도 먼저 나서서 하려고 해주는 덕분에 미안하고도 고마웠습니다. 앞으로 열심히 해서 좋은 연구원이 됐으면 좋겠습니다.

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