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Antifibrotic Effects of High Mobility
Group Box 1 Protein Inhibitor
(Glycyrrhizin) on Keloid Fibroblasts
and Keloid Spheroids through
Reduction of Autophagy and
Induction of Apoptosis

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Directed by Professor Won Jai Lee

The Doctoral Dissertation
submitted to the Department of Medicine
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of Doctor of Philosophy

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ABSTRACT

Antifibrotic Effects of High Mobility Group Box 1 Protein Inhibitor (Glycyrrhizin) on Keloid Fibroblasts and Keloid Spheroids through Reduction of Autophagy and Induction of Apoptosis

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Keloids are considered benign fibroproliferative tumors. Overabundance of extracellular matrix resulting from hyperproliferation of keloid fibroblasts (KFs), and dysregulation of apoptosis represent the main pathophysiological mechanisms underlying the formation of keloids. High-mobility group box 1 (HMGB1) is considered relevant in various diseases as it plays important roles in inflammation, mitogenesis, and the regulation of cell death. Suppression of intracellular HMGB1 expression inhibits autophagy, which predominantly serves as a cell survival mechanism, whilst increasing apoptotic cell death. Suppression of HMGB1 with glycyrrhizin has therapeutic benefits in fibrotic diseases. In this study, we explore the possible involvement of autophagic activity and HMGB1 as a cell death regulator in keloid pathogenesis. We highlight the potential utility of glycyrrhizin as an antifibrotic agent via the regulation of the aberrant balance between autophagy and apoptosis in keloids.

The expression of HMGB1 in keloid and normal dermal tissue was

investigated by immunohistochemistry (IHC). Transmission electron microscopic examination was performed to detect autophagosomes in both KFs and HDFs. Further, IHC for Beclin 1 and light chain protein 3 (LC3), which are markers of autophagy, was performed in tissue samples. Using an autophagy defect kit, the basal autophagy levels in KFs, HDFs, transforming growth factor- β (TGF- β)-treated HDFs, and HMGB1-treated HDFs were investigated. After treatment of KFs and human dermal fibroblasts (HDFs) with glycyrrhizin (0, 100, 200, and 500 μ M), cell proliferation was assessed. Apoptosis as well as autophagic activity were determined in keloids treated with glycyrrhizin. Expression of major ECM components and factors associated with fibrosis were investigated in keloid spheroids treated with glycyrrhizin. Levels of autophagy and collagen accumulation in the TGF- β -treated fibrotic condition were examined following the application of the autophagy inhibitor 3-methyladenine (3-MA).

Higher HMGB1 expression was observed in keloid than in normal tissue. Additionally, high autophagic activity was found in KFs as well as in keloid tissue. In TGF- β - or HMGB1-treated fibrotic condition, an enhanced autophagy level was detected in HDFs. Decreased HMGB1 expression was confirmed in keloid spheroids treated with glycyrrhizin. The proliferation of KFs was decreased following glycyrrhizin treatment. While the level of autophagy was decreased, the rate of apoptosis was increased in KFs following glycyrrhizin application. The expression levels of profibrogenic molecules, namely ERK1/2, Akt, and NF- κ B, were enhanced in HMGB1-treated HDFs but significantly decreased following glycyrrhizin treatment. The expression levels of types I and III collagen, fibronectin, and

elastin were reduced in glycyrrhizin-treated keloid spheroids. TGF- β , Smad2/3, ERK1/2, and HMGB1 were decreased significantly in keloid spheroids following the application of glycyrrhizin. Enhanced autophagy was detected in KFs and keloid tissue. While enhanced apoptosis was detected in keloids after glycyrrhizin treatment, autophagy markers were significantly reduced. Treatment with the autophagy inhibitor 3-MA resulted in a marked decrease in the levels of autophagy markers and collagen expression in the TGF- β -induced fibrotic condition.

The results indicate that autophagy plays an important role in the pathogenesis of keloids. Because the HMGB1 blocker, glycyrrhizin, appears to degrade ECM and downregulate autophagic cell death in keloids, its potential use for treatment of keloids is indicated.

Key words : Autophagy, HMGB1, glycyrrhizin, keloid

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I. INTRODUCTION

Keloids, considered benign fibroproliferative tumors that culminate in abnormal dermal fibrosis, are characterized by the excessive deposition of extracellular matrix (ECM), chiefly collagen fibers. Generally, these tumors invade adjacent normal tissue and rarely regress spontaneously¹. In recent years, there has been increasing interest in the field of pathologic scars and several mediators have been found to influence the pathogenesis of keloids, albeit without a clear understanding of the underlying mechanism. An overabundance of ECM resulting from uncontrolled proliferation of keloid fibroblasts (KFs) is one of the most well-known causative factors involved in keloid development²⁻⁷. Thus, it has commonly been assumed that keloid formation is caused by increased cellular proliferation and reduced rate of apoptosis in KFs⁸⁻¹⁰. Appropriate therapies may be directed at either inhibiting proliferation of KFs or reversing pathologic fibrosis.

Autophagy is a highly conserved cellular death process involving

degradation of cell components via lysosomal degradation. This contributes to the maintenance of cellular homeostasis by degrading and recycling unnecessary or impaired cellular components¹¹. In particular, autophagy has been considered an adaptive pro-survival mechanisms in cell subject to cellular stress such as nutrient deprivation, prolonged inflammation, hypoxia, or anti-cancer treatment. Autophagy is therefore associated with various human pathophysiologies, such as fibrosis of internal organs, aging, tissue remodeling and neurodegenerative disease¹². Extensive research has been carried out on the importance of autophagy in cellular homeostasis, especially under stress; however, no single study that adequately covers the action of autophagy in pathologic dermal fibrosis, such as keloid formation, has been reported to date. As autophagy promotes cellular viability even under stressed conditions, we speculate that dysregulated cellular death in keloids is associated with uncontrolled proliferation of KFs and development of keloids. The first key research question of this study was whether autophagic activity is altered in keloids.

High-mobility group box 1 (HMGB1) is an ubiquitous nuclear protein that acts as a DNA chaperone participating in DNA replication, recombination, transcription, and repair¹³. Upon cellular activation or injury, HMGB1 translocates outside the nucleus, and is released into the cytosol or extracellular space. Overexpressed cytosolic HMGB1 is associated with increased cellular proliferation, mobility, angiogenesis, and resistance to apoptosis whilst promoting autophagy and inflammation¹⁴⁻¹⁹. Thus, extracellular HMGB1 functions as a damage-associated molecular pattern (DAMP) protein that activates the inflammatory response, promoting cellular

proliferation, differentiation and migration²⁰. All of these processes contribute to tumorigenesis as well as to pathologic fibrosis. Accordingly, recent evidence suggests that HMGB1 is involved in chronic inflammation, cancer, and various fibrotic diseases^{13,14,21-27}. Thus, HMGB1 has been regarded as a key regulator of autophagy, as both cytosolic HMGB1 and extracellular HMGB1 enhance autophagic activity in response to cellular stress^{19,28}. As extranuclear HMGB1 promotes cell survival under stressed conditions by inducing autophagy, we sought to determine if HMGB1 is associated with keloid pathogenesis through regulation of the cellular death process. Thus, we hypothesized that the inhibition of autophagic activity while inducing apoptosis may exert therapeutic effects on keloids.

It has been shown that glycyrrhizin, which is extracted from the licorice root, directly binds to HMGB1 and inhibits its chemotactic and mitogenic activities in the extracellular space. Further, this compound has been shown to inhibit the cytoplasmic translocation of HMGB1^{29,30}. The protective effects of glycyrrhizin have been demonstrated in inflammation, pathologic fibrosis, and oncogenesis^{29,31-35}. Consequently, inhibition of HMGB1 with glycyrrhizin may disrupt keloid progression and attenuate fibrosis in keloids.

In this study, we focused on the autophagic activity of keloids in regulating fibrogenesis along with possible involvement of HMGB1. In addition, we highlighted the potential of glycyrrhizin, a potent inhibitor of HMGB1, as a promising agent for the treatment of keloids.

II. MATERIALS AND METHODS

1. Preparation of cells

Normal human dermal fibroblasts (HDFs) and KFs were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), penicillin (30 U/mL), and streptomycin (300 µg/mL). Cultures were maintained at 37°C in a humidified incubator under 5% CO₂, changing the medium every 2 days. In all experiments, cells were used before passage #7.

2. Preparation of keloid spheroids

Keloid and adjacent normal dermal tissues were obtained during surgical procedure from patients with active-stage keloids after having obtained informed consent from each subject (n = 5, Table 1). Keloid spheroids were prepared as described previously³⁶ by dissecting keloid central dermal tissue into 2-mm-diameter pieces with sterile 21-gauge needles. Explants were plated onto HydroCell® 24 multi-well plates (Nunc, Rochester, NY, USA) and cultured for 4 hr in Iscove's modified Dulbecco's medium (Gibco) supplemented with 5% fetal bovine serum, 10 µM insulin, and 1 µM hydrocortisone. Glycyrrhizin, an inhibitor of HMGB1, was added into the plates containing keloid spheroids at 0, 100, 200, or 500 µM, and incubated at 37°C in 5% CO₂ for 3 days. The treated keloid spheroids were then fixed with 4% formalin, paraffin-embedded, and cut into 5-µm-thick sections.

Table 1. Demographics of keloids. Demographic information and description of keloids obtained from study subjects.

	Sex	Race	Age (years)	Origin
1	F	Korean	18	Ankle
2	F	Korean	33	Earlobe
3	M	Korean	4	Neck
4	M	Korean	31	Neck
5	F	Korean	11	Knee

3. Histology and immunohistochemical assessment of tissue

Keloid and normal tissues were fixed in 10% buffered formalin and embedded in paraffin blocks followed by sectioning at 5- μ m thickness. Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). Immunohistochemistry (IHC) was conducted to detect HMGB1 and autophagy markers. Paraffin sections were deparaffinized, rehydrated, and incubated with citric acid for antigen retrieval. Tissues were blocked with 2% bovine serum albumin and incubated with the following primary antibodies: HMGB1 (Abcam, Cambridge, MA, USA), light chain protein 3 (LC3) (Cell Signaling Technology, Danvers, MA, USA), and Beclin 1 (Abcam). After washing with phosphate-buffered saline (PBS), the slides were incubated with a secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To assess the influence of glycyrrhizin in autophagy markers, IHC of Beclin 1

and LC3 were performed after treated with 0, 200 or 500 μ M of glycyrrhizin. Expression levels of autophagy factors were quantitated using computer-assisted planimetry (Metamorph®, Universal Image, Buckinghamshire, UK).

4. Transmission electron microscopy (TEM)

Cultured HDFs and KFs were pre-fixed with 2% glutaraldehyde (Merck, Boston, MA, USA), 2% paraformaldehyde (Merck), and 0.5% CaCl_2 (Sigma-Aldrich). Sections were then washed with 0.1 M phosphate buffer (pH 7.4) and post-fixed with 1% OsO_4 in 0.1 M phosphate buffer (Polysciences, Warrington, PA, USA). After post-fixation, blocks were ultra-thin-sectioned, and the sections were double stained with uranyl acetate (6%) and lead citrate. Sections were observed at 5,000 x and 25,000 x using a JEM-1011 transmission electron microscope (JEOL, Pleasanton, CA, USA).

5. Flow cytometric analysis of autophagy and apoptosis

Autophagic activity of the cells was measured using a Cyto-ID® autophagy detection kit (Enzo Life Sciences, Farmingdale, NY, USA) following the manufacturer's protocol. In brief, KFs, HDFs, and HDFs treated with 10 ng of TGF- β (Sigma-Aldrich) for 48 hr (1×10^4 cells/cm², each) were trypsinized and collected by centrifugation. The cells were washed with PBS and stained with Cyto-ID® Green dye for each sample. After 30 min of incubation at room temperature in the dark, cells were washed and collected by centrifugation. Resuspended cell pellets were analyzed using the green channel of a flow cytometer. The same procedures were performed in HDFs and 100 ng of

HMGB-1 (Sigma-Aldrich)-treated HDFs for 48 hr.

After treatment of HDFs and KFs (1×10^4 cells/cm²) for 48 hr with 0, 100, 200, or 500 μ M glycyrrhizin, an Annexin V-FITC assay was performed to evaluate the extent of apoptosis. KFs and HDFs were dissociated with trypsin, washed once with PBS, and then with the Annexin V binding buffer (1 \times). The cells were stained with 5 mL of Annexin V-FITC at room temperature for 15 min, counterstained with propidium iodide (1 mg/mL), and analyzed using a CyAnTMADP flow cytometer (Dako).

6. Histology and immunohistochemical assessment of keloid spheroid

Keloid spheroids were treated with 0, 100, 200, or 500 μ M of glycyrrhizin for 48 hr. The spheroids were then washed, fixed with 4% formalin, paraffin-embedded, and cut into 5- μ m-thick sections. Representative sections were stained with Picrosirius red, and then examined by light microscopy. For IHC staining, the keloid spheroid sections were incubated at 4°C overnight with mouse anti-HMGB1 (Abcam), mouse anti-collagen type I (Abcam), mouse anti-collagen type III (Sigma-Aldrich), mouse anti-elastin (Sigma-Aldrich), mouse anti-fibronectin (Santa Cruz Biotechnology), rabbit anti-TGF- β (Abcam), rabbit anti-ERK1/2 (Cell Signaling Technology), or rabbit anti-Smad2/3 primary antibodies. Sections were then incubated at room temperature for 20 min with the EnvisionTM kit (Dako, Glostrup, Denmark) as a secondary antibody. Diaminobenzidine/hydrogen peroxidase (Dako) was used as the chromogen substrate. All slides were counterstained with Meyer's hematoxylin. The expression levels of HMGB1, type I collagen, type III collagen, elastin, fibronectin, TGF- β , ERK1/2, and Smad2/3 were

semi-quantitatively analyzed using Metamorph® image analysis software. Results are expressed as the mean optical density of six different digital images.

7. Methylthiazolyldiphenyl-tetrazolium bromide assay

To assess cellular viability after glycyrrhizin treatment in KFs and HDFs, Methylthiazolyldiphenyl-tetrazolium bromide assay was performed: 1×10^4 cells/cm² of KFs and HDFs were seeded in triplicate 96 wells. After exposing the cells for 48 hr to 0, 500 μ M, 1 mM, and 2 mM of glycyrrhizin (Sigma-Aldrich), 200 μ L of a 0.5 mg/mL MTT solution (Boehringer, Mannheim, Germany) was added to each well and plates were incubated at 37°C for 3 hr. To dissolve the resulting formazan, 200 μ L of dimethyl sulfoxide (Sigma-Aldrich) was added to each well after the MTT solution was removed. Absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

8. Western blotting of autophagy markers and profibrotic markers

Quantitative measurement of representative autophagy markers, LC3-I, LC3-II and Beclin 1, was performed using western blotting. HDFs, KFs, and KFs treated with 200 μ M glycyrrhizin (cells were seeded at 10^5 cells/well) were cultured in 100 mm \times 20 mm dishes for 48 hr. Cells were lysed in 50 mM Tris-HCl (pH 7.6), 1% Nonidet P-40, 150 mM NaCl, and 0.1 mM zinc acetate in the presence of protease inhibitors. Protein concentrations were determined by the Lowry method (Bio-Rad), and 20 μ g of each sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Proteins were then electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was blocked with blocking buffer for 1 hr and then incubated overnight at 4°C with primary antibodies against LC3-I, LC3-II, Beclin 1, and actin (mouse monoclonal; Sigma-Aldrich). After 2 hr of incubation at room temperature with the secondary antibody (horseradish peroxidase-conjugated anti-rabbit or anti-mouse; Santa Cruz Biotechnology), protein bands were visualized using chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. Protein expression was analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Quantitative measurement of representative profibrotic markers, ERK1/2, Protein kinase B (Akt), Nuclear factor-kappa B (NF- κ B) also carried out using western blot analysis: 1×10^5 HDF cells were seeded per well, and treated with 100 ng of HMGB1 or 100 ng of HMGB1 with 200 μ M of glycyrrhizin for 48 hr (non-treated HDFs vs. HMGB1-treated HDFs vs. HMGB1 + glycyrrhizin-treated HDFs (n = 3/each group)). Western blotting of profibrotic markers of non-treated HDFs, HMGB1-treated HDFs and HMGB1-with-glycyrrhizin-treated HDFs was performed with primary antibodies against with ERK1/2, Akt (Cell Signaling Technology), NF- κ B (Cell Signaling Technology), and actin (mouse monoclonal; Sigma-Aldrich). All other steps were as described above.

Quantitative assessment of autophagy markers was performed using western blotting in TGF- β treated HDFs after autophagy inhibitor application. 1×10^5 HDF cells were seeded per well, and treated with 10 ng of TGF- β with

various concentrations of 3-methyladenine [3-MA (Sigma, Saint Louis, Mo); 0, 2.5, 5 μ M] for 48 hr. Western blotting of autophagy markers of TGF- β -treated HDFs, TGF- β with 2.5 μ M of 3-MA- treated HDFs and TGF- β with 5 μ M of 3-MA-treated HDFs was performed with primary antibodies against LC3-I and II, Beclin 1, and actin [TGF- β -treated HDFs vs. TGF- β + 2.5 μ M of 3-MA-treated HDFs vs. TGF- β + 5 μ M of 3-MA-treated HDFs (n = 3/each group)]. All other steps were as described above.

9. In vivo terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

Keloid spheroids were treated with 0, 100, 200, or 500 μ M of glycyrrhizin for 48 hr. The spheroid sections were deparaffinized and treated with proteinase K (20 μ g/mL) for 15 min. Endogenous peroxidases were blocked using 3% hydrogen peroxide in PBS for 10 min. Samples were washed and incubated at room temperature with terminal deoxynucleotidyl transferase buffer. Excess buffer was drained and the tissue was incubated for 1 hr at 37°C with terminal transferase and biotin-16-dUTP. Tissues were then rinsed four times with PBS and incubated for 1 hr at 37°C a 1:400 dilution of peroxidase-conjugated streptavidin. Slides were rinsed with PBS and incubated for 5 min with 3,3'-diaminobenzidine. Sections were then washed three times with PBS and counterstained with methyl green. The rate of apoptosis was semi-quantitatively analyzed using MetaMorph® image analysis software. Results are expressed as the mean optical density for five different digital images.

10. RNA analysis of type I and III collagen

To evaluate mRNA levels of type I and III collagen in TGF- β treated HDFs after autophagy inhibitor application, quantitative real-time PCR (qRT-PCR) was performed 48 hr after treat with 10 ng TGF- β (Sigma, Saint Louis, Mo) or cotreated TGF- β and 3-MA (2.5 or 5 μ M). Total RNA was prepared with the RNeasy Mini Kit (Qiagen, Hilden, Germany), and complementary DNA was prepared from 0.5 μ g of total RNA by random priming using a first-strand cDNA synthesis kit (AccuPower™ RT PreMix, Bioneer, Daejeon, Korea) under the following conditions: 42°C for 60 min, 95°C for 5 min. Applied Biosystems TaqMan primer/probe kits were used to analyze mRNA expression levels with an ABI Prism 7500 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For cDNA amplification, AmpliTaq Gold DNA polymerase was activated by 10 min incubation at 95°C; this was followed by 40 cycles of 15 s at 95°C and 1 min at 60°C for each cycle. The mRNA expression levels were normalized to the levels of the GAPDH housekeeping gene, and then relative quantities were expressed as fold-inductions compared with the control gene after determining the threshold cycle and drawing standard curves. The primers were described in table 2.

Table 2. List of primers used in this study.

Gene	TaqMan assay ID
COL1A1	Hs00164004_m1
COL3A1	Hs00164103_m1
Human GAPDH	Hs99999905_m1

11. Statistical analysis

Results are expressed as means \pm standard error of the mean (SEM). Data were analyzed by a repeated-measures one-way ANOVA. Paired t-test was used to analyze statistical differences between two groups. Results were judged significant when $p < 0.05$.

III. RESULTS

1. Expression of HMGB1 in keloid tissue

Recent findings suggest that HMGB1 is important in various forms of tissue fibrosis^{16,17,21,37-39}. HMGB1 is a ubiquitous nuclear protein in eukaryotic cells. In stressful environments, nuclear HMGB1 translocates to the cytosol and is released into the extracellular space. To determine the collagen deposition pattern and HMGB1 expression level in keloid tissue and normal dermis, H&E staining and IHC of HMGB1 were performed on keloid tissue as well as on adjacent normal dermal tissue. H&E staining indicated a multidirectional woven meshwork of normal collagen structure in adjacent normal dermis (Figure 1a), and densely packed thick hyalinised collagen fibers in keloid tissue (Figure 1b). IHC of HMGB1 revealed that the expression of HMGB1 was remarkably increased in keloid tissue compared with that in normal dermis (Figure 1c-e). In particular, a high-power view showed that HMGB1 was abundantly expressed in the cytosol and extracellular space of keloid tissue (Figure 1d, 400 x magnification).

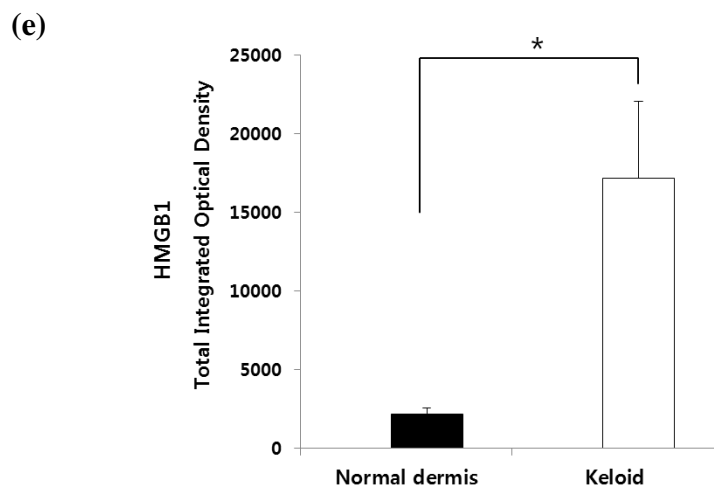
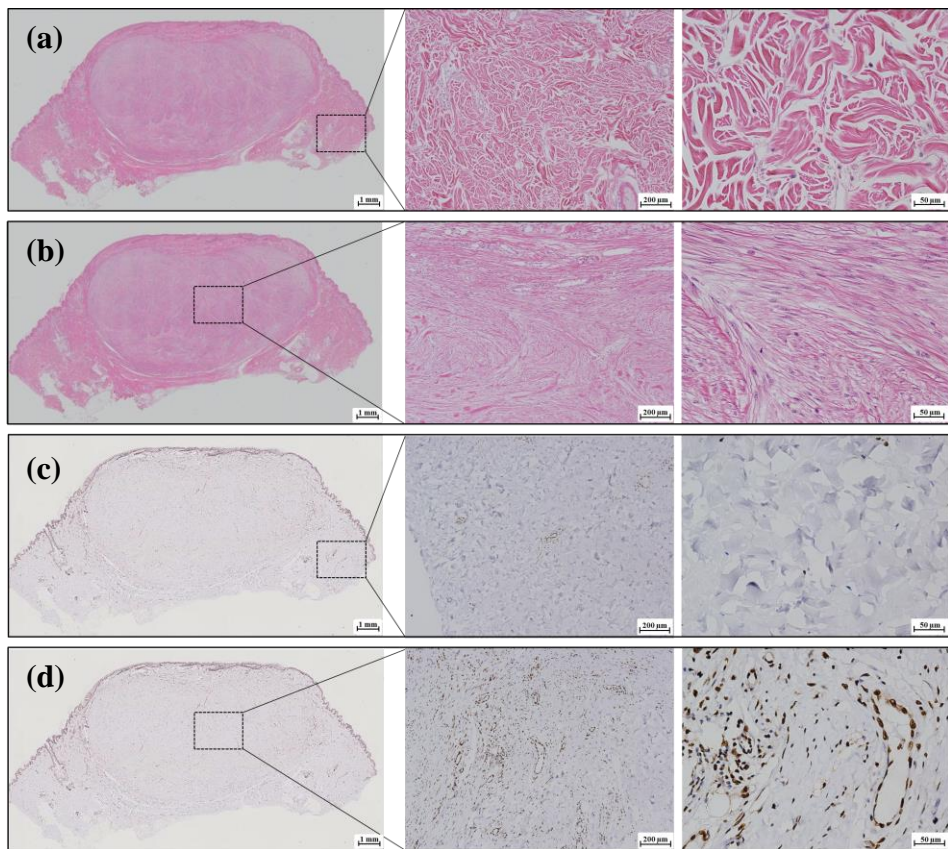


Figure 1. H&E and histochemical analysis of HMGB1. (a and b) In H&E staining, densely accumulated thick collagen bundles were noted in keloid tissue. (c and d) In IHC of HMGB1, excessive expression of HMGB1 was noted in keloid tissue while expression of HMGB1 was rarely seen in normal dermis. (e) Semi-quantitative analysis indicated that the expression of HMGB1 was significantly increased in keloid tissue compared with that in normal dermal tissue (* $p < 0.05$ vs. normal dermis). Original magnification 100 x, 400 x.

2. Detection of autophagosomes in keloid fibroblasts

Although basal autophagic activity is generally low under normal conditions, it may increase under various stressful conditions. Further, HMGB1 is a crucial regulator of autophagy: extranuclear HMGB1 triggers autophagy, which induces extranuclear HMGB1 translocation^{19,40}. To verify whether the basal autophagy level is increased in keloids, KFs as well as HDFs were ultrastructurally examined by TEM. Detection of autophagosomes, which are defined as double-membrane structures enclosing undigested cytoplasmic contents, is a standard method for the detection of autophagy⁴¹⁻⁴⁴. The low-power view of electron micrographs showed increased numbers of autophagosomes containing electron-dense materials in the cytoplasm of KFs relative to HDFs (magnification 5,000 x, Figure 2a and c; arrow: autophagosomes). The ultrastructure of double-membraned autophagic vacuoles was confirmed at highly magnified view of cells (magnification 25,000 x, Figure 2b and d). The experiments were repeated three times with similar results and representative images are shown (Figure 2).

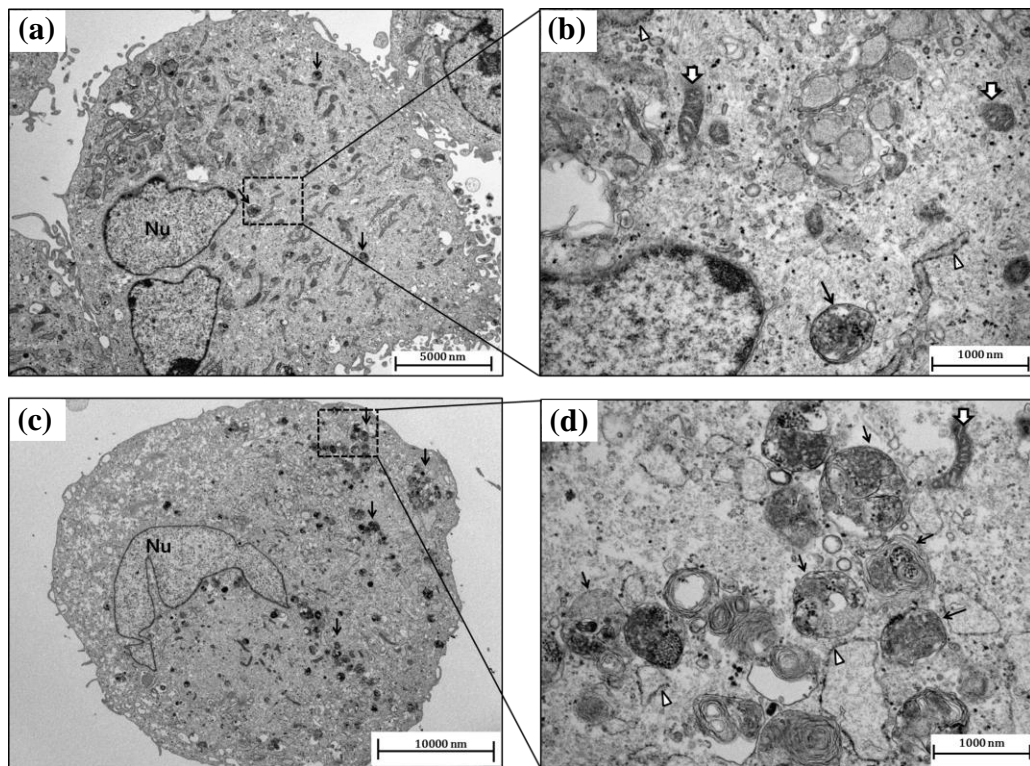


Figure 2. Transmission electron microscopic analysis of keloid fibroblasts. Comparison of basal autophagy levels between KFs and HDFs was performed by detecting autophagosomes. (a) Low-power view of HDFs: several intracellular organelles including rough endoplasmic reticulum (ER), mitochondria, Golgi apparatus, and a few autophagosome (arrow) are visible in the cytoplasm. (b) High-power view of HDFs: an autophagosome (arrow) containing degraded double-membrane-bound organelles, rough ER (arrow head) and mitochondria (open arrow) is visible. (c) Low-power view of KFs; several intracellular organelles including rough ER, mitochondria, Golgi apparatus, and an increased number of autophagosomes (arrow) are visible in the cytoplasm. (d) High-power view of KFs: several autophagosomes (arrow), rough ER (arrow head), and mitochondria (open arrow) are visible. Each experiment was performed in triplicate. Representative data are shown (Nu- nucleus).

3. Autophagy markers in keloid tissue

To determine the basal autophagy level in keloids, at both the cellular and tissue level, IHC of Beclin 1 and the microtubule-associated protein, LC3, which are widely used for monitoring autophagy was performed (Figure 3a)⁴⁵. Compared with the adjacent normal dermal tissue, expression levels of Beclin 1 significantly increased in both clinical keloid margin (the transitional region) and keloid tissue by 3.2 times and 2.9 times, respectively ($***p < 0.001$ vs. normal dermis, Figure 3b). The expression of LC3 in transitional area and keloid tissue are also increased by 5.4 times and 6.1 times, respectively, compared with that in normal dermis ($***p < 0.001$ vs. normal dermis, Figure 3c). In each experiment, no significant differences existed between transitional region and keloid tissue for autophagy markers. This corresponds to the ultrastructural findings of TEM analysis, which showed that autophagic activity is enhanced not only in KFs but also in keloid tissue.

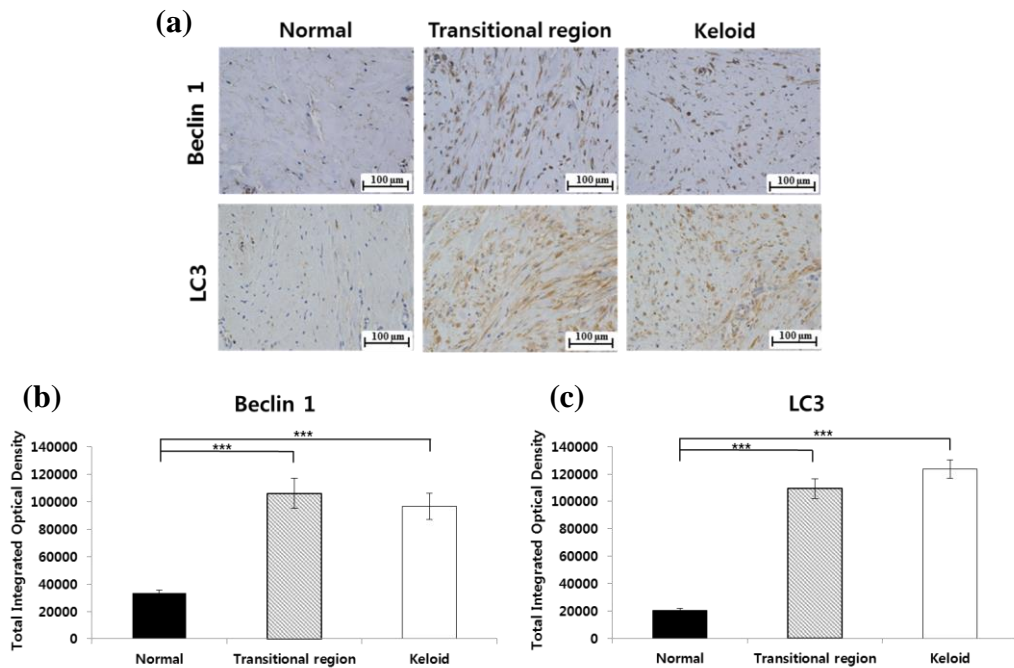
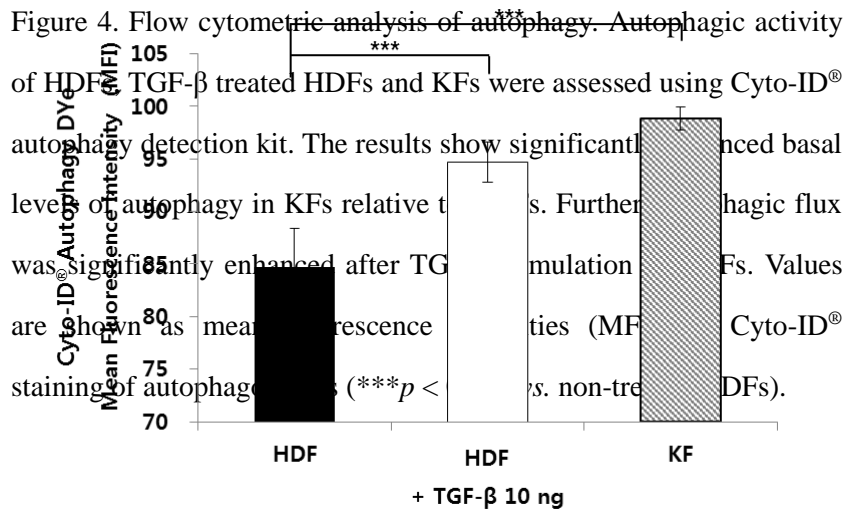


Figure 3. Histochemical analysis of autophagy markers in keloid tissue and normal dermal tissue. Comparison of basal autophagy levels between keloid tissue and adjacent normal dermis was performed using IHC of Beclin 1 and LC3. (a) Note particularly high levels of autophagy markers in the keloid and transitional regions of keloids. (b and c) Semi-quantitative analysis showed that the expression of Beclin 1 and LC3 were significantly increased in keloid tissue and clinical keloid margin (transitional region) compared with that in normal tissue. The differences were statistically significant (***) $p < 0.001$ for the indicated comparisons). Data are expressed as mean \pm SEM of six experiments.

4. Autophagy levels of keloid fibroblasts and TGF- β -treated normal human dermal fibroblasts

To further validate basal autophagy levels in KFs, HDFs, and TGF- β -treated HDFs, we performed autophagy assay using a Cyto-ID[®] autophagy dye. This dye is a cationic amphiphilic tracer that selectively labels autophagic vacuoles and enables quantification of autophagic activity by flow cytometry. The results confirmed a significantly enhanced autophagic activity in KFs, with a 1.17-fold increase observed between HDFs. These findings are consistent with our previous results indicating enhanced basal levels of autophagy in keloids. TGF- β is a key cytokine known to stimulate fibrosis. In order to replicate fibrotic conditions, we treated HDFs with 10 ng of TGF- β and compared the autophagic activity with that in non-treated HDFs. The result showed significantly enhanced autophagic activity in TGF- β -treated HDFs, with a 1.12-fold increase compared with that in non-treated HDFs (Figure 4). Collectively, these data imply that autophagy is enhanced in fibrotic conditions of human dermal skin such as keloids.



5. Autophagy levels of HMGB1-treated normal human dermal fibroblasts

We postulated that enhanced and prolonged release of extranuclear HMGB1 contributes to the development of pathologic fibrosis by increasing autophagic activity in HDFs. Accordingly, we examined whether exogenous HMGB1 induces autophagy in HDFs. The level of autophagic flux of non-treated

HDFs and HDFs treated with 100 ng of HMGB1 was analyzed by flow cytometry using a Cyto-ID® autophagy detection kit. As shown in Figure 5, autophagic activity of HMGB1-treated HDFs was higher than that of non-treated HDFs by 1.29 fold (** $p < 0.01$). These data suggest that exogenous HMGB1 induces autophagic cell death in HDFs. As HMGB1 is highly expressed in keloids, these findings led us to hypothesize that

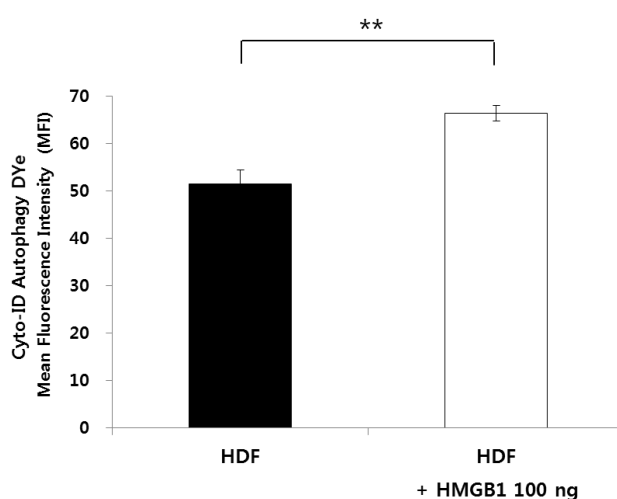


Figure 5. Flow cytometric analysis of autophagy after treatment of HMGB1. Autophagic activity of HDFs and 100 ng of HMGB1-treated HDFs was analyzed with a Cyto-ID® autophagy detection kit. The results show that exogenous HMGB1 induces autophagic cell death in HDFs. Values were shown as mean fluorescence intensities (MFI) for Cyto-ID® staining of autophagosomes (** $p < 0.01$ vs. non-treated HDFs).

extracellular HMGB1 enables cellular survival by enhancing autophagic cell death in keloids.

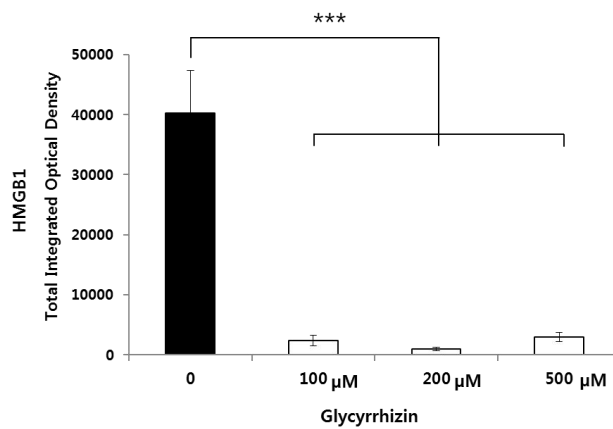
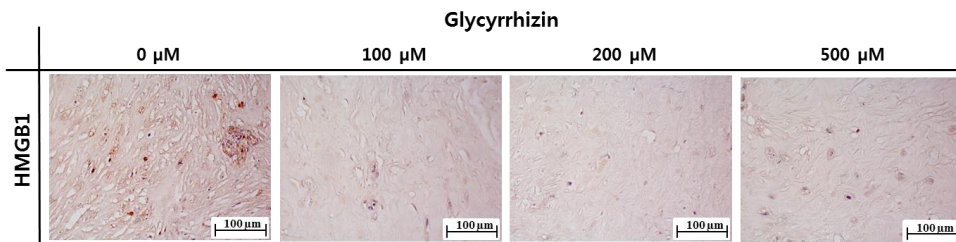
6. Effect of glycyrrhizin on HMGB1 expression in keloid spheroids

If exogenous HMGB1 promotes autophagic activity in keloids, then inhibition of HMGB1 should reduce cellular viability of keloids. We assessed this hypothesis by treatment with glycyrrhizin, which binds directly to HMGB1 and suppresses its extracellular activities as well as inhibits cytoplasmic translocation of HMGB1. Although glycyrrhizin is recognized as a potent HMGB1 inhibitor, no study has investigated the effect of this compound on keloids. Therefore, we used keloid spheroids to assess whether glycyrrhizin could reduce HMGB1 expression in keloids. We generated keloid spheroids following an established protocol³⁶ to mimic the keloid microenvironment. After treatment of keloid spheroids with various concentrations of glycyrrhizin (0, 100, 200, or 500 μ M), IHC staining for HMGB1 was performed. As shown in Figure 6a, non-treated keloid spheroids showed higher expression of HMGB1, while glycyrrhizin-treated keloid spheroids showed markedly decreased HMGB1 expression. Data are graphed using Metamorph[®] image analysis software. The results show significantly decreased HMGB1 expression in keloid spheroids treated with 100, 200, and 500 μ M of glycyrrhizin, by 93.9%, 97.7%, and 92.7% respectively, versus non-treated keloid spheroids ($***p < 0.001$, Figure 6b).

(a)

(b)

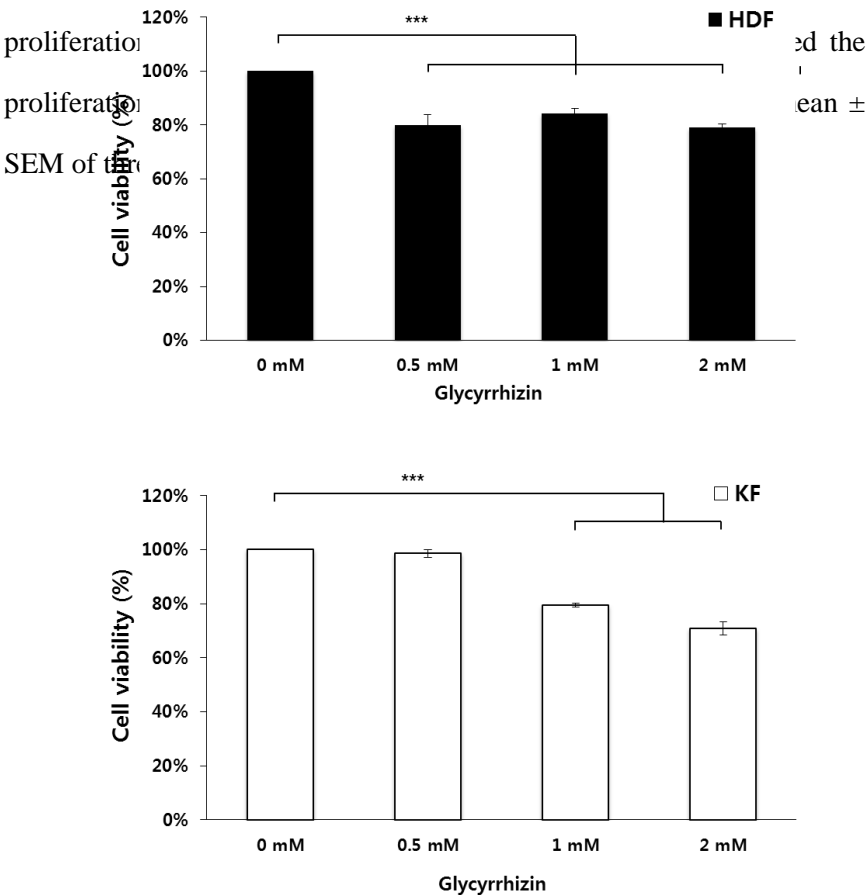
Figure 6. Effect of glycyrrhizin on HMGB1 expression in keloid spheroids. (a) IHC was used to identify HMGB1 in keloid spheroids. Following the addition of glycyrrhizin, the density of the HMGB1-positive area was notably decreased in keloid spheroids. (b) Semi-quantitative analysis indicated significantly decreased HMGB1 in glycyrrhizin (100, 200, and 500 μ M)-treated keloid spheroids versus non-treated keloid spheroids. Data are expressed as mean \pm SEM of six experiments (***) $p < 0.001$).



7. Effect of glycyrrhizin on viability of keloid fibroblasts

To determine whether inhibition of HMGB1 with glycyrrhizin affects keloidal cell viability, we performed an MTT assay. To assess cellular viability after glycyrrhizin treatment, various concentrations of glycyrrhizin (0 mM, 0.5 mM, 1 mM, or 2 mM) were applied to KFs and HDFs 48 hr before the MTT assay. The results showed a significant decrease in KF and HDF proliferation after treatment with all tested concentrations of glycyrrhizin (Figure 7). These

Figure 7. Effect of glycyrrhizin on viability of HDFs and KFs. MTT cell

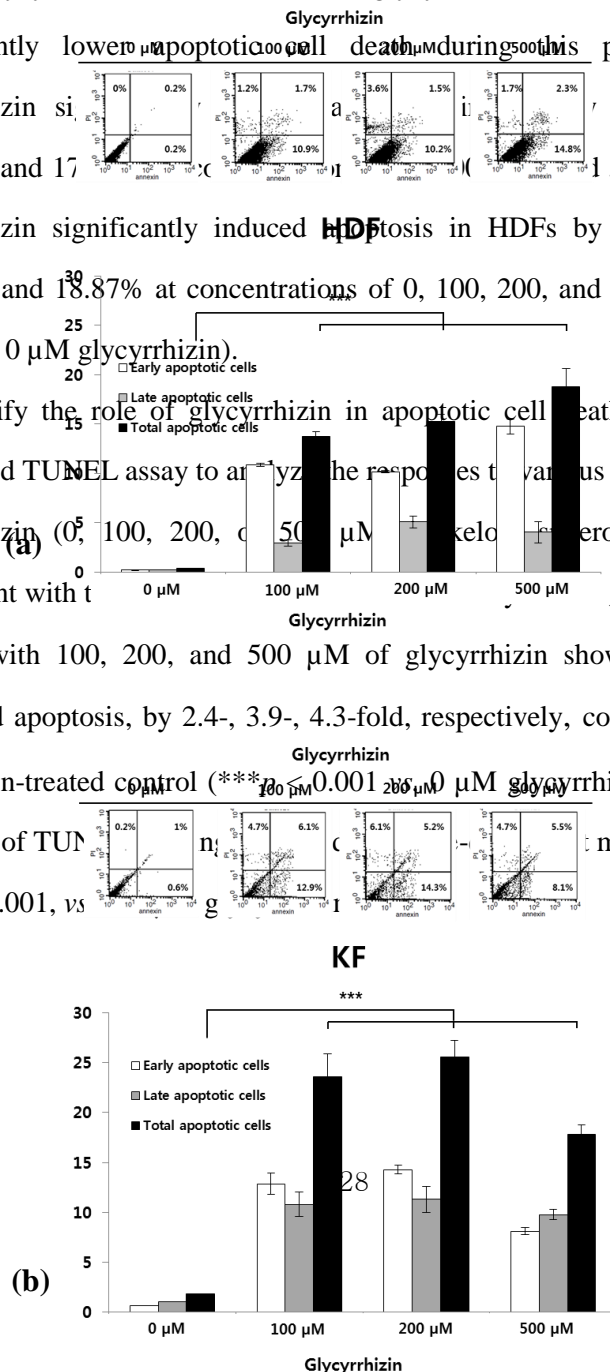


results suggest that glycyrrhizin reduces cellular viability of keloids.

8. Effect of glycyrrhizin on apoptosis of keloid fibroblasts and keloid spheroids

To explore whether glycyrrhizin induces apoptosis in keloids, HDFs and KFs were treated with 0, 100, 200, or 500 μM of glycyrrhizin. As shown in Figure 8a and b, HDFs and KFs both underwent increased apoptotic cell death 48 hr after glycyrrhizin treatment. Non-glycyrrhizin-treated KFs showed significantly lower apoptotic cell death during this period; however, glycyrrhizin significantly induced apoptosis in HDFs by 0.35%, 13.76%, 15.24%, and 18.87% at concentrations of 0, 100, 200, and 500 μM (** $p < 0.001$ vs. 0 μM glycyrrhizin).

To clarify the role of glycyrrhizin in apoptotic cell death of keloids, we performed TUNEL assay to analyze the responses to various concentrations of glycyrrhizin (0, 100, 200, and 500 μM) in keloid spheroids (Figure 8c). Consistent with the results in HDFs, keloid spheroids treated with 100, 200, and 500 μM of glycyrrhizin showed significantly increased apoptosis, by 2.4-, 3.9-, 4.3-fold, respectively, compared with that in the non-treated control (** $p < 0.001$ vs. 0 μM glycyrrhizin). Further, the intensity of TUNEL staining increased in a dose-dependent manner (Figure 8d, ** $p < 0.001$, vs. 0 μM glycyrrhizin).



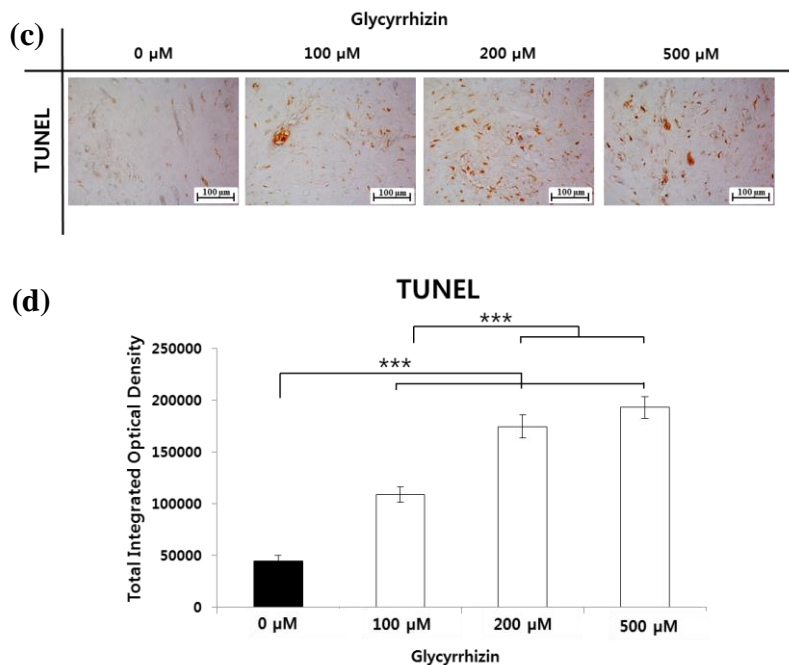


Figure 8. Apoptosis assay of keloid fibroblasts and keloid spheroids after treatment of glycyrrhizin. (a and b) The Annexin V-FITC assay show that glycyrrhizin induces apoptosis in HDFs and KFs ($***p < 0.001$ vs. 0 μ M glycyrrhizin). (c) Representative images of keloid spheroids stained by TUNEL for apoptosis assay. TUNEL-positive cells dose-dependently increased after glycyrrhizin treatment. (d) The TUNEL assay show enhanced apoptotic activity in glycyrrhizin-treated keloid spheroids compared with that in the non-treated control. Data are expressed as mean \pm SEM of five experiments ($***p < 0.001$).

9. Effect of glycyrrhizin on autophagy of keloid fibroblasts and keloid tissue

To assess the consequence of glycyrrhizin-induced autophagic cell death in keloids, we examined changes in Beclin 1 expression and conversion rate of LC3-I to LC3-II in both fibroblast cell types by western blot. The western blot results indicated that the levels of Beclin 1 and LC3-II/I conversion rate in KFs were markedly higher, by 1.4 and 1.3 fold, in comparison with that in HDFs. Further, the significantly enhanced levels of Beclin 1 and LC3-II/I found in KFs, compared with that in HDFs, were notably decreased following treatment with 200 μ M of glycyrrhizin ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Figure 9a-c). This result is concordant with IHC data for keloid tissue that revealed a significant decrease of Beclin 1 and LC3 after glycyrrhizin treatment (Figure 9d). Beclin 1 and LC3 expression levels in keloid tissues treated with glycyrrhizin (500 μ M) were significantly reduced by 18.1% and 24.6%, respectively, in comparison with that in non-treated keloid tissue ($***p < 0.001$, Figure 9e-f).

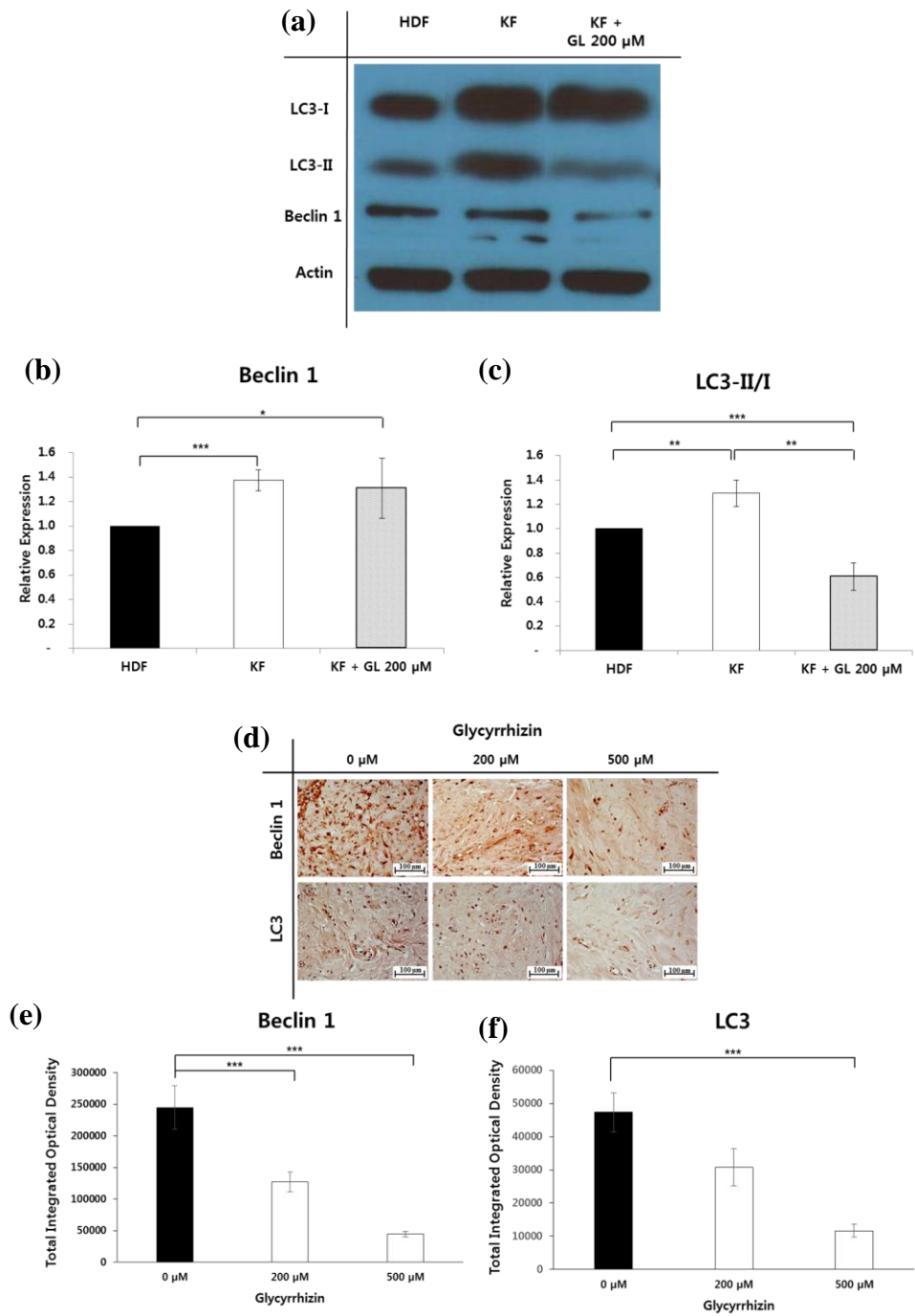


Figure 9. Autophagy assay of keloid fibroblasts and keloid tissue after treatment of glycyrrhizin. (a) Western blot analysis of autophagy markers in HDFs, KFs, and 200 μ M of glycyrrhizin-treated KFs are shown. (b and c) Beclin 1 and LC3 levels are increased in KFs compared with that in HDFs. KFs show significantly decreased after treatment with 200 μ M of glycyrrhizin. (d) IHC for autophagy markers in keloid tissue after glycyrrhizin treatment (0, 200, 500 μ M). (e and f) Semi-quantitative analysis revealed that the expression levels of Beclin 1 and LC3 were dose-dependently reduced in glycyrrhizine-treated keloid tissue ($***p < 0.001$ vs. 0 μ M glycyrrhizin). (GL- glycyrrhizin)

10. Effect of HMGB1 and glycyrrhizin on the expression of profibrotic factors in human dermal fibroblasts

We speculate that upregulated extranuclear HMGB1 serves as a profibrotic molecule by increasing cellular proliferation, producing large amounts of ECM, and stimulating profibrogenic factors. Our previous results have demonstrated that autophagic cell death is increased in keloids, and that exogenous fibrogenic molecules, such as TGF- β and HMGB1, enhance autophagic activity in HDFs. Following this, we attempted to determine whether exogenous HMGB1 increases profibrotic molecules in fibroblasts and enhances the activity of glycyrrhizin. Profibrogenic signaling molecules involved in collagen synthesis and cellular proliferation, such as ERK1/2, Akt, and NF- κ B, were assessed by western blot analysis. We found significant

changes in the expression levels of these molecules in HMGB1-treated HDFs. As shown in Figure 10, treatment with 100 ng of HMGB1 resulted in significantly increased expression of ERK1/2, Akt, and NF- κ B. Interestingly, markedly decreased expression of all of the factors was observed after simultaneous treatment with glycyrrhizin (200 μ M) and HMGB1 (100 ng) (* p < 0.05; Figure 10). These results indicate that exogenous HMGB1 induces profibrotic signaling molecules, and that glycyrrhizin reverse the action of HMGB1.

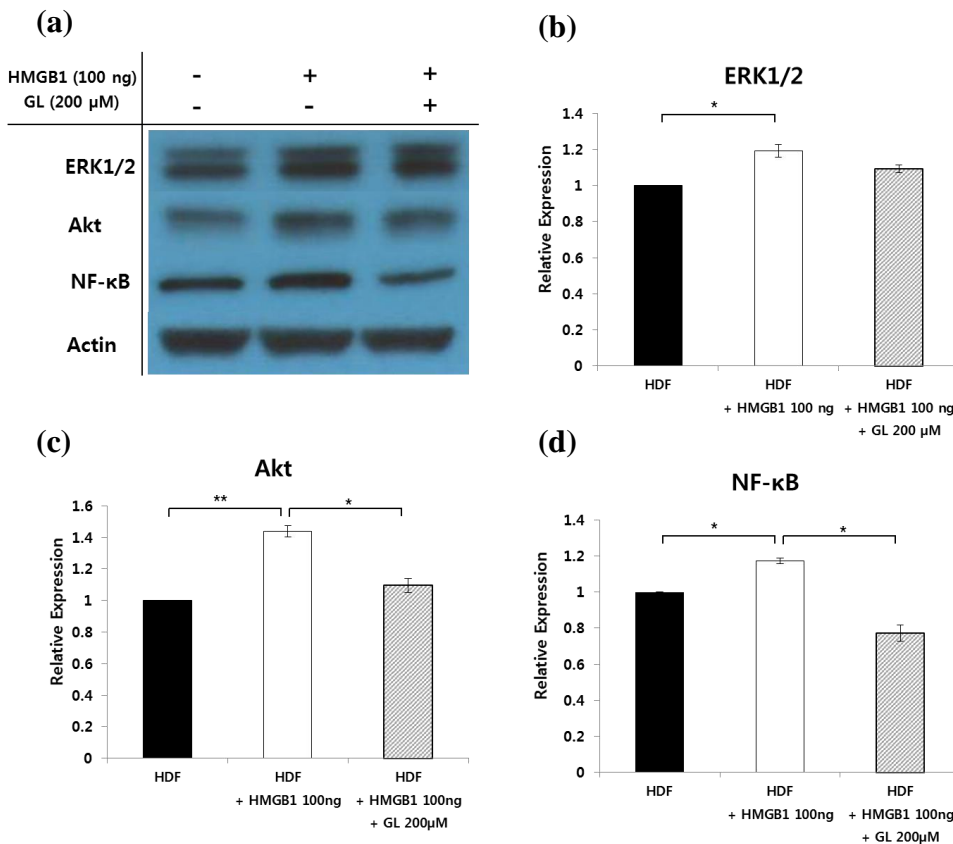
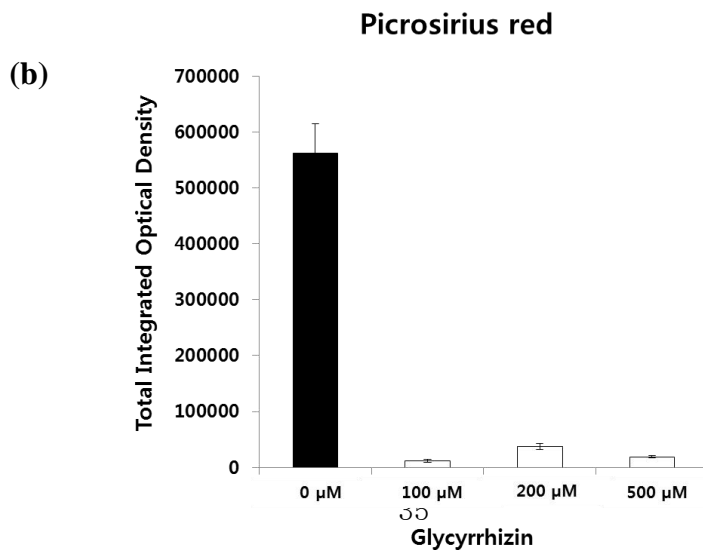
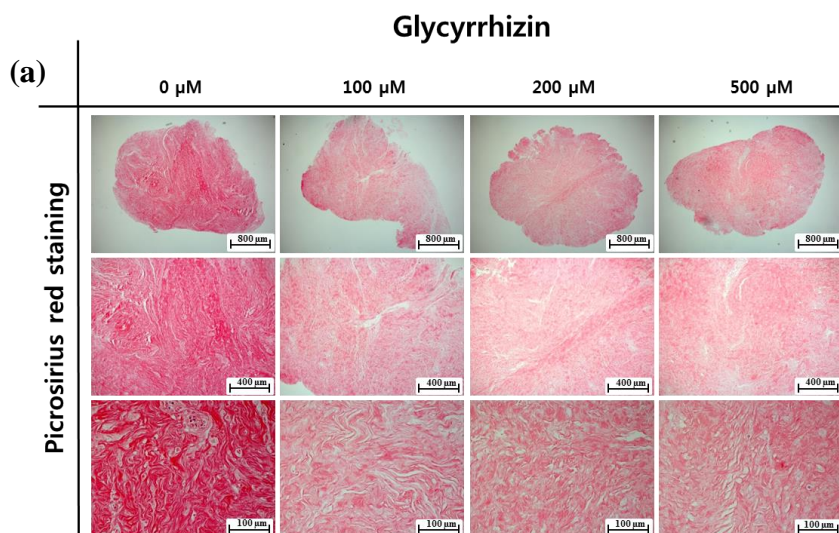


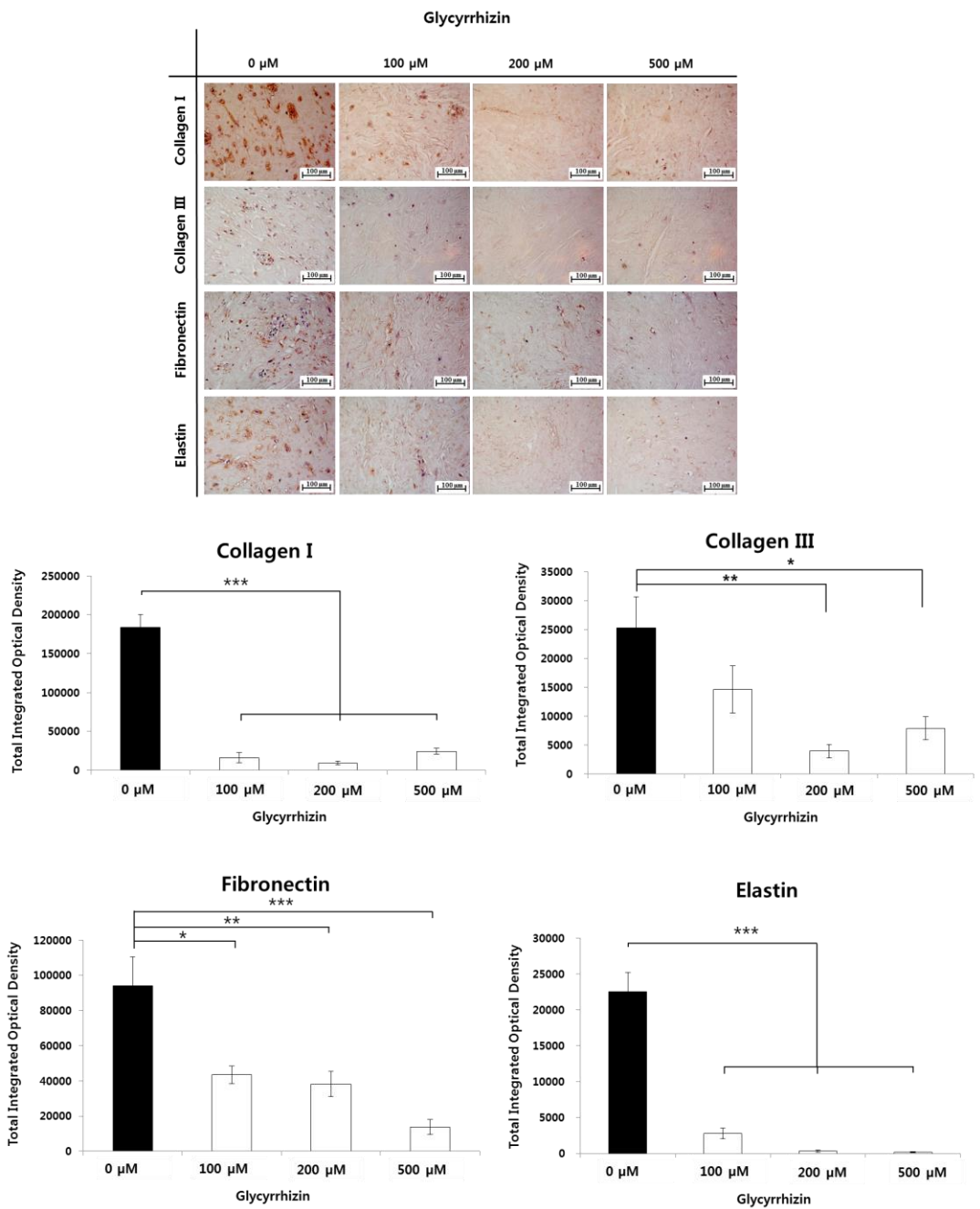
Figure 10. Effect of HMGB1 and glycyrrhizin on the expression of profibrotic factors in human dermal fibroblasts. (a-d) ERK1/2, Akt, and NF- κ B expression was significantly increased in the HMGB1 (100 ng)-treated HDFs. However, the enhanced profibrotic factors markedly decreased after glycyrrhizin (200 μ M) treatment simultaneously with HMGB1 (100 ng) (* p < 0.05, ** p < 0.01). (GL- glycyrrhizin)

11. Effect of glycyrrhizin on the expression of extracellular matrix components in keloid spheroids

We next determined whether glycyrrhizin reduced fibrosis in keloids. Histological visualization of collagen with Picrosirius red stain revealed densely packed collagen bundles in keloid spheroid. However, excessively accumulated thick, coarse collagen fibers were markedly decreased in keloid spheroids after treatment with glycyrrhizin (Figure 11a). Imaging analysis of IHC showed significantly decreased collagen deposition in glycyrrhizin (100, 200, and 500 μ M)-treated keloid spheroids by 97.9%, 93.3%, and 96.6%, respectively, compared with that in the untreated control (*** p < 0.001, Figure 11b). Correspondingly, there was a considerable decrease in the expression of typical ECM components, including type I and III collagen, fibronectin, and elastin, in glycyrrhizin-treated keloid spheroids (Figure 11c). As shown in Figure 11 d-g, the addition of glycyrrhizin (200 μ M)

significantly reduced the levels of type I collagen, type III collagen, fibronectin, and elastin, by 95.0%, 84.3%, 59.4%, and 98.5% respectively, compared with that in non-treated keloid spheroids. These data suggest that the most representative ECM components, type I collagen, type III collagen, fibronectin, and elastin, were significantly reduced following administration of glycyrrhizin to keloid spheroids.





(c)

Figure 11. Histochemical analysis of the ECM component of glycyrrhizin-treated keloid spheroids. (a) Picrosirius red staining show coarse, densely packed collagen bundles in keloid spheroids, which decreased in density after glycyrrhizin treatment. (b) Semi-quantitative measurements reveal significantly decreased collagen deposition in keloid spheroids after treatment with 100, 200, and 500 μ M glycyrrhizin ($***p < 0.001$ vs. 0 μ M glycyrrhizin). (c) Representative images and quantitative analysis of type I collagen (d), type III collagen (e), fibronectin (f) and elastin (g) in glycyrrhizin-treated keloid spheroid. Markedly decreased expression of ECM components was observed. Data are expressed as mean \pm SEM of six experiments ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

12. Effect of glycyrrhizin on the expression of TGF- β , and its signaling pathway in keloid spheroids

We further investigated the effect of glycyrrhizin on TGF- β , Smad2/3, and ERK1/2 expression; these molecules are crucial regulators of fibrogenesis. IHC staining revealed significantly reduced TGF- β , Smad2/3, and ERK1/2 levels in glycyrrhizin (200 μ M)-treated keloid spheroids, by 78.1%, 94.7% and 93.7%, respectively, versus non-treated keloid spheroids ($***p < 0.001$, Figure 12). Collectively, these data suggest that glycyrrhizin modulates TGF- β and its signaling pathway, thus reducing fibrosis in keloids.

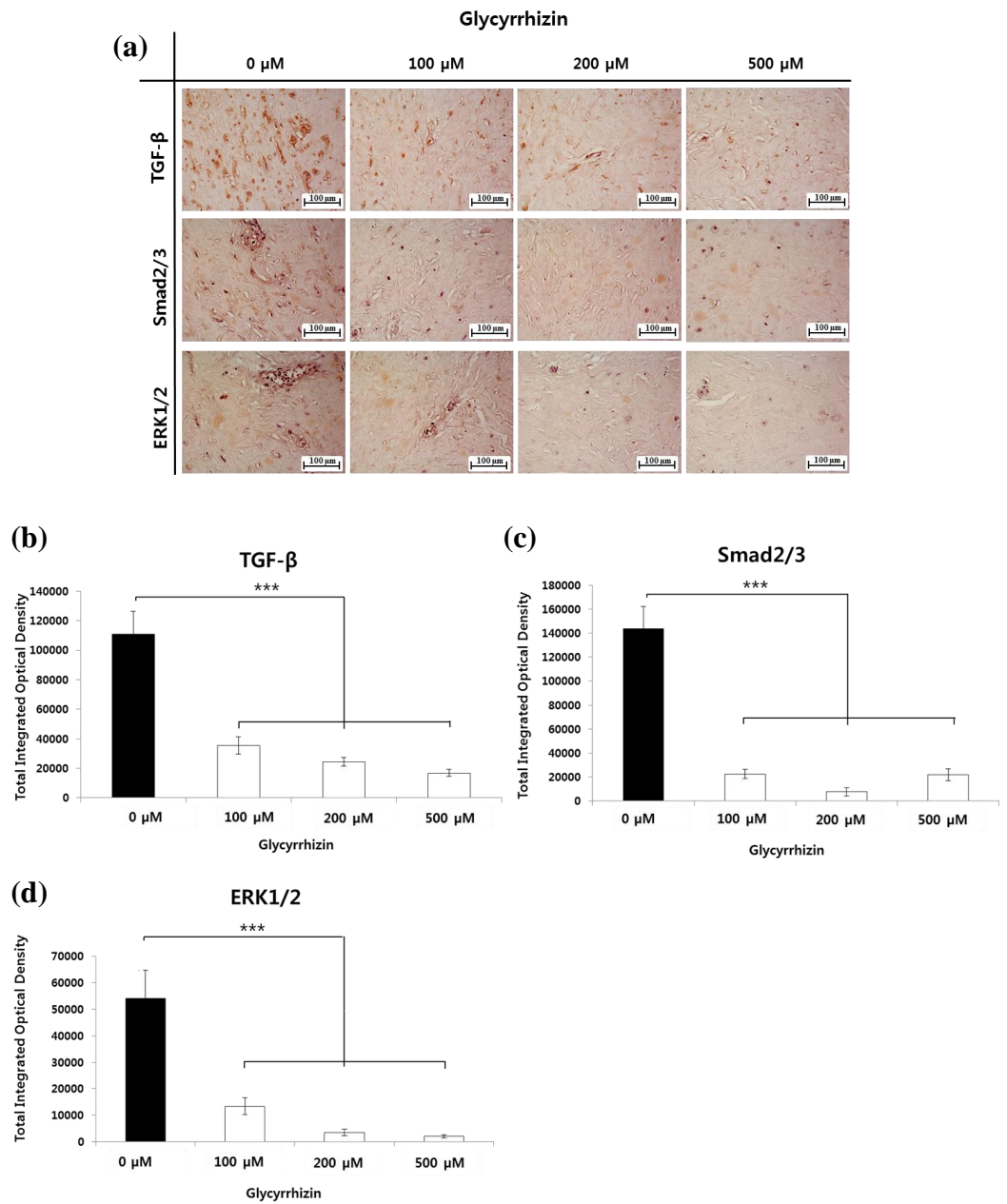


Figure 12. Histochemical analysis of TGF- β , Smad2/3, and ERK1/2 in glycyrrhizin-treated keloid spheroids. (a) Representative images of TGF- β , Smad2/3, and ERK1/2 IHC of keloid spheroids treated with glycyrrhizin (0, 100, 200, or 500 μ M). (b-d) TGF- β , Smad2/3, and ERK1/2 were significantly decreased in keloid spheroids following glycyrrhizin application. The data shown are representative of six independent experiments (*** $p < 0.001$ vs. 0 μ M glycyrrhizin).

13. Effect of 3-methyladenine on collagen accumulation in fibrotic condition

Here, we demonstrated that glycyrrhizin inhibits cellular proliferation by enhancing apoptosis, decreasing autophagic activity, and reducing fibrosis in keloids. The next question we examined was whether inhibiting autophagy reduces fibrosis in fibrogenic conditions. As shown in Figure 4, we demonstrated enhanced autophagic flux in TGF- β treated HDFs. Under the same fibrotic conditions, we evaluate the influence of autophagy inhibitor 3-MA on autophagy level and collagen expression in TGF- β treated HDFs. We treated HDFs with 10 ng of TGF- β and various concentrations of 3-methyladenine (3-MA; 0, 2.5, 5 μ M) for 48 hr and performed western blot analysis. The conversion ratios of representative autophagy marker LC3-II/I and Beclin 1, were significantly reduced in the 3-MA-treated group compared with that in the non-treated group, suggesting a dose-dependent effect. However, there were no significant difference between the groups treated with

2.5 μM and 5 μM of 3-MA (** $p < 0.01$, Figure 13a-c). Notably, significant decrease of type I and III collagen expression were observed in TGF- β -treated HDFs following the addition of 5 μM 3-MA (** $p < 0.001$, Figure 13d and e). Therefore, the results show that type I and III collagen, which are the main components of scar tissue, were significantly reduced following the inhibition of autophagic activity in fibrotic dermal conditions.

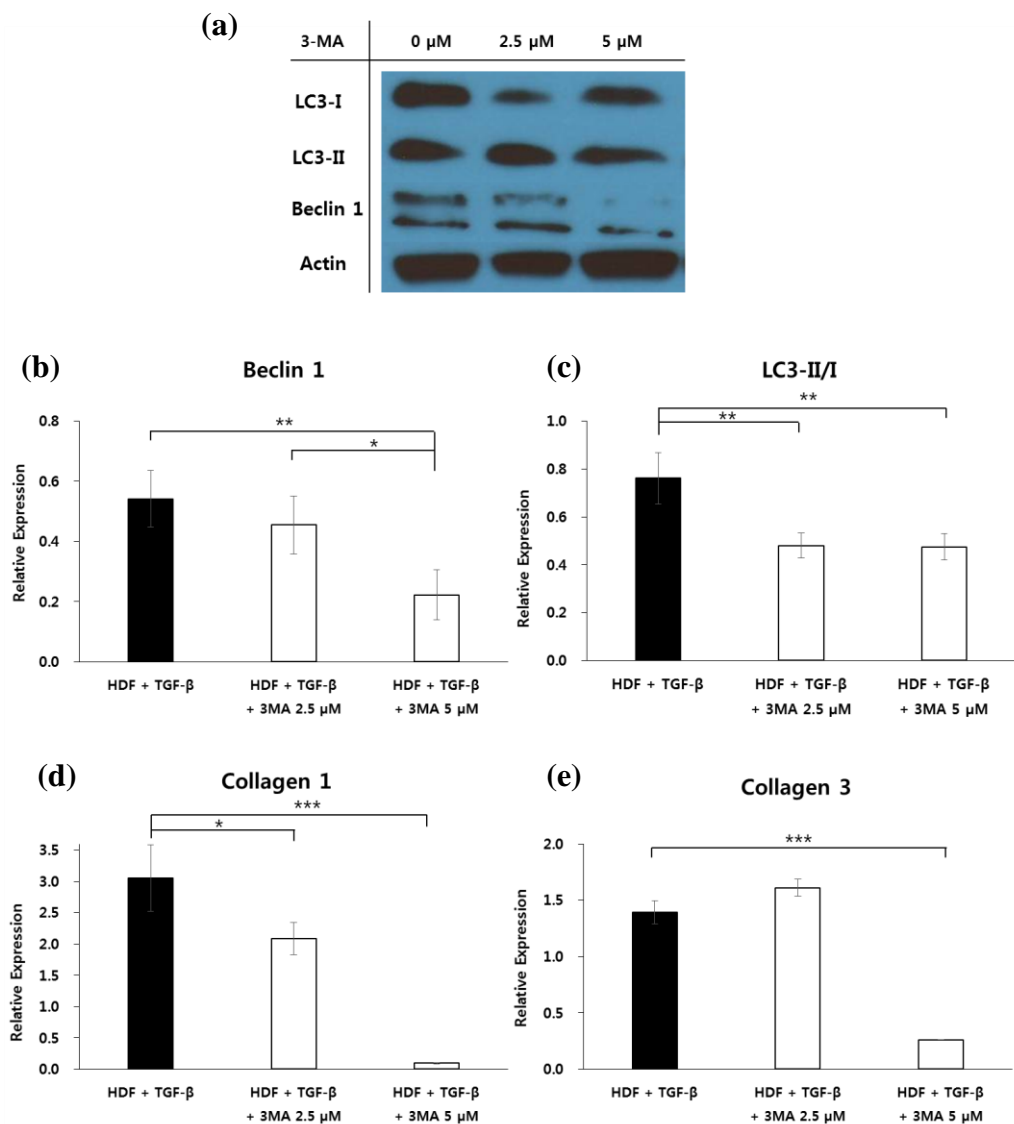


Figure 13. Effect of 3-methyladenine on TGF- β -treated human dermal fibroblasts. (a-c) Expression of Beclin 1 and LC3-II/I was significantly decreased in TGF- β (10 ng)-treated human dermal fibroblasts following autophagy inhibitor, 3-methyladenine (3-MA), application ($*p < 0.05$, $**p < 0.01$ vs. 0 μ M 3-MA). (d and e) Deposition of type I and type III collagen were markedly reduced in TGF- β (10 ng)-treated human dermal fibroblasts following the application of 5 μ M 3-MA ($***p < 0.001$ vs. 0 μ M 3-MA).

IV. DISCUSSION

Keloids, which represent a human fibrotic disorder characterized by dermal fibroproliferative tumors, extend beyond the boundaries of the original scar and invade adjacent normal skin. Although various factors influence the development of keloids, excessive extracellular matrix accumulation resulting from hyperproliferation of KFs and dysregulation of apoptosis are one of the main pathophysiological factors involved^{5-7,46}.

Autophagy is a form of cell death that involves lysosomal degradation and recycling of damaged or excess organelles. Although autophagy is a cellular death process, an increasing body of evidence supports that this process also acts as a cytoprotective mechanism that ensures adequate energy metabolism under conditions of stress such as starvation, oxidative stress, hypoxia, and anticancer therapy^{19,47-49}. Therefore, we hypothesized that enhanced autophagic activity is associated with the development of keloids. Using TEM, we detected notably enhanced autophagosomes in KFs and increased

expression of the autophagy markers, Beclin 1 and LC3, in keloid tissue and KFs. The results confirm the hypothesis that keloids are associated with high autophagic activity. These results differ from those of a previous study that report decreased Beclin 1, LC3-I, and LC3-II in hypertrophic scar tissue⁵⁰. In various microenvironments, both increased and decreased autophagy play vital roles in the pathogenesis of diseased tissue¹². Although keloid and hypertrophic scar tissue appear clinically similar, their molecular bases and clinical behaviors are quite different; for example, they exhibit different apoptotic cell death pathways and distinct sensitivities to KF growth factors^{51,52}.

HMGB1, a ubiquitous and abundant nuclear protein, has chemotactic and mitogenic activities in inflammatory cells and fibroblasts^{53,54}. Emerging evidence suggests that HMGB1 is involved in pathologic fibrosis affecting various organs of the human body including the heart, liver, lung and kidney, as well as tumorigenesis, via the modulation of inflammation, tissue fibrosis, immune responses, and cell death^{14-16,21,23,38,55}. Cytoplasmic translocation of HMGB1 promotes autophagy and limits programmed apoptotic cell death. Endogenous HMGB1 regulates the balance between apoptosis and autophagy^{11,56}. Cellular stress promotes HMGB1 release from cells and the released HMGB1 promotes autophagic flux^{19,28}. Therefore, we speculated that HMGB1 is associated with aberrant cellular death in keloids that exhibit attenuated apoptotic activity^{8,57}. Accordingly, we confirmed the presence and overexpression of HMGB1 in human keloid tissues. Further, enhanced autophagic activity was confirmed in HDFs treated with exogenous HMGB1 or TGF- β to induce fibrotic conditions. Subsequently, we inhibited HMGB1

activity and observed changes in cell death and factors related to fibrogenesis in keloids.

Recent evidence has shown that glycyrrhizin, which binds directly to HMGB1 to impair extracellular activity and inhibit its extracellular release, decreases the chemoattractant and mitogenic activities of HMGB1^{29,31,33,35}. Here, we showed that glycyrrhizin attenuated HMGB1 expression and suppressed mitogenic activity in keloids. Further, we demonstrated that the enhanced expression of representative autophagy markers, Beclin 1 and LC3, in KFs was reduced by glycyrrhizin treatment, while apoptotic cell death was enhanced not only in KFs but also in keloid tissue. These findings suggest that glycyrrhizin attenuates cellular proliferation by enhancing apoptosis, reducing autophagy, and reversing the aberrant cellular death process in keloids. Consistent with previous work using glycyrrhizin as a HMGB1 inhibitor in fibrotic diseases⁵⁸⁻⁶⁰, we also demonstrated that this compound ameliorates fibrosis in keloid spheroids.

TGF- β is a crucial factor in proliferation and collagen synthesis in keloids as it enhances the mitogenic response^{61,62}. Pivotal mediators of the TGF- β signaling pathway, the Smad2/3 and ERK1/2 complexes, are highly activated in keloids and have been implicated in keloid pathogenesis⁶¹⁻⁶⁴. We found that the expression of TGF- β , and the Smad2/3 and ERK1/2 complexes, were significantly attenuated by glycyrrhizin in keloid spheroids. Together, these results reveal that glycyrrhizin exerts a potent anti-fibrotic effect on keloids.

The direct inhibitory effect of glycyrrhizin on HMGB1 is already well known^{16,17}, and inhibitory effects of the profibrogenic action of exogenous HMGB1 were demonstrated in this study. Further, glycyrrhizin possesses

various pharmacological and biological activities against inflammation, oxidative stress, and tumorigenesis, suggesting that the present effects may not be solely attributable to the inhibitory effect of HMGB1. Further, the inhibition of autophagic activity in keloids was not the only contributing factors to the anti-fibrotic action of glycyrrhizin. However, we demonstrated that inhibition of autophagic activity with 3-MA elicits a significant reduction of collagen deposition under fibrotic condition.

Additional studies with other inhibitory molecules of HMGB1, such as ethyl pyruvate or anti-HMGB1 monoclonal antibodies, are needed to further verify that HMGB1 is involved in pathologic dermal fibrotic disorders such as keloids.

The present study, to our knowledge, is the first to demonstrate enhanced autophagic cell death in keloids. The HMGB1 blocker, glycyrrhizin, was shown to ameliorate fibrosis in keloids. This effect may result from the inhibition of TGF- β -related pathways as well as regulation of the cell death process.

V. CONCLUSION

Autophagic activity is enhanced in keloids. Inhibition of HMGB1 with glycyrrhizin decreases fibrosis, increases apoptosis, and diminishes autophagy in keloids. These results suggest that targeting HMGB1-mediated fibrosis as well as autophagy represents a novel strategy for the treatment of keloids.

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ABSTRACT(IN KOREAN)

켈로이드에서 High Mobility Group Box 1 Protein 억제제인
Glycyrrhizin의 자가포식작용 및 세포 사멸사 조절을 통한
항섬유 작용

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전여름

Keloid는 발생기전 및 치료방법이 명확히 밝혀지지 않은 섬유성 피부 병변으로 비정상적으로 세포의 증식이 증가되고 사멸사가 감소하며 과도하게 세포외기질이 축적되는 것을 특징으로 한다. High mobility group box 1 (HMGB1)은 대부분의 진핵 세포에 존재하는 핵 단백질로 염증, 면역, 세포의 증식 및 사멸 등을 조절하여 다양한 섬유성 병변과 관련되어 있는 것으로 알려져 있다. 자가포식은 에너지 결핍 등의 스트레스 조건에서 세포가 생존할 수 있도록 돕는 세포 사멸의 한 방법으로, HMGB1은 세포사멸과 자가포식간 균형을 조절하는 것으로 알려져 있다. 이에 우리는 자가포식이 keloid의 병인과 관계되어 있지 않은지 확인해보고자 하였다. 또한, HMGB1의 세포외 작용을 억제하는 것으로 알려진 glycyrrhizin을 이용하여 keloid에서 HMGB1과 glycyrrhizin이 세포 사멸의 균형에 미치는 영향과, 이를 통한 항섬유 효과를 확인해 보고자 하였다.

면역화학염색을 통해 정상조직에 비해 keloid 조직에서 HMGB1의 발현이 증가함을 확인하였고, 투과전자현미경을 이용해 자가포식소체가 켈로이드에서 증가되어있으며, 켈로이드 섬유모세포

및 조직에서 자가포식 표지인자인 Beclin 1과 LC3이 증가되어 있음을 확인하였다. 유세포분석을 통해 대표적 섬유성 인자인 TGF- β 와 HMGB1을 처리한 섬유모세포에서 자가포식이 증가함을 확인하였다. 켈로이드 세포구에 glycyrrhizin을 처리하여 HMGB1의 발현이 감소함을 면역화학염색을 통해 확인하였으며, MTT 분석을 통해 켈로이드 섬유모세포와 정상 섬유모세포에서 glycyrrhizin (0, 100, 200, 500 μ M) 처리후 세포의 증식이 유의하게 감소함을 확인하였다. 자가포식 표지인자의 면역화학염색을 통해 glycyrrhizin의 처리에 따라 자가포식이 유의하게 감소하나, 유세포분석 및 TUNEL 검사에서 세포자멸은 유의하게 증가하는 결과를 확인하였다. Western blot을 통해 HMGB1을 처리한 섬유모세포에서 ERK1/2, Akt, NF- κ B가 유의하게 증가하나 glycyrrhizin을 처리 후 다시 감소함을 확인하였다. 또한 켈로이드 세포구에서 type 1, 3 collagen, fibronectin, elastin과 TGF- β , Smad2/3, ERK1/2의 발현이 glycyrrhizin 처리에 따라 유의하게 감소함을 western blot을 통해 확인하였다. TGF- β 를 처리한 정상 섬유모세포에 자가포식 억제제인 3-methyladenine을 처리해 자가포식이 감소하고, 교원질의 발현도 감소함을 PCR검사를 통해 확인하였다. 이 결과들을 토대로, 켈로이드에서 자가포식이 증가되어 있고, HMGB1의 억제제인 glycyrrhizin의 처리에 따라 자가포식이 억제되고, 세포자멸이 증가하며, 섬유화가 감소하는 결과를 확인하였다. 이를 통해 keloid의 억제 및 치료방법 개발에 응용할 수 있을 것으로 사료된다.

핵심되는 말 : 자가포식, High Mobility Group Box 1 (HMGB1), 글리시리진, 켈로이드