# Anti-apoptotic effects of glycosaminoglycans via inhibition of ERK/AP-1 signaling in TNF-α-stimulated human dermal fibroblasts

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Received August 23, 2017; Accepted February 7, 2018

DOI: 10.3892/ijmm.2018.3483

**Abstract.** It has been established that glycosaminoglycans (GAGs) serve an important role in protecting the skin against the effects of aging. A previous clinical trial by our group identified that a cream containing GAGs reduced wrinkles and increased skin elasticity, dermal density and skin tightening. However, the exact molecular mechanism underlying the anti-aging effect of GAGs has not yet been fully elucidated. The present study assessed the influence of GAGs on cell viability, collagen synthesis and collagen synthesis-associated signaling pathways in tumor necrosis factor-α (TNF-α)-stimulated human dermal fibroblasts (HDFs); an *in vitro* model of aging. The results demonstrated that GAGs restored type I collagen synthesis and secretion by inhibiting extracellular signal-regulated kinase (ERK) signaling in TNF-α-stimulated HDFs. However, GAGs did not activate c-jun N-terminal kinase or p38. It was determined that GAGs suppressed the phosphorylation of downstream transcription factors of ERK activation, activator protein-1 (AP-1; c-fos and c-jun), leading to a decrease in matrix metalloproteinase-1 (MMP-1) levels and the upregulation of tissue inhibitor of metalloproteinase-1 in TNF-α-stimulated HDFs. In addition, GAGs attenuated the apoptosis of HDFs induced by TNF-α. The current study revealed a novel mechanism: GAGs serve a crucial role in ameliorating TNF-α-induced MMP-1 expression, which causes type I collagen degeneration via the inactivation of ERK/AP-1 signaling in HDFs. The results of the present study indicate the potential application of GAGs as effective anti-aging agents that induce wrinkle reduction.

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*Key words:* glycosaminoglycans, type I collagen, matrix metalloproteinase-1, extracellular signal-regulated kinase, apoptosis

## Introduction

Collagen degradation is the primary cause of skin aging. The modulation of collagen synthesis/degradation is thus a pivotal target for anti-wrinkle agents. Glycosaminoglycans (GAGs) are highly charged polysaccharides that are an important structural component of the extracellular matrix (ECM) and are often used in cosmetic products (1-3). In a previous clinical trial, it was identified that a cream containing GAGs regulated wrinkles, skin elasticity, dermal density and skin tightening (4). However, although GAGs serve important roles in skin aging, their regulatory mechanism of action has not yet been fully elucidated.

Skin wrinkles develop as a result of intrinsic and extrinsic aging processes (5,6). Intrinsic aging, which is characterized by a smooth, thinned epidermis that exhibits fine wrinkles, naturally occurs over time and is dependent on the accumulation of inflammatory mediators. Extrinsic aging, characterized by a roughened texture, skin laxity and mottled pigmentation with deep wrinkles, occurs due to solar irradiation, smoking and poor nutrition (7,8). These alterations may also be caused by ECM destruction as a dermal fibroblast aging that occur during intrinsic and extrinsic aging (9). Dermal fibroblasts serve an important role in the production of ECM, including the production of ground substance, collagen fibers and elastins (10). Therefore, the disruption of fibroblast function affects the mechanical properties of skin connective tissue. Inflammatory mediators, including cytokines, serve a crucial role in stimulating skin aging (11). The expression of a gene cluster associated with inflammation (12) and the quantity of halogenated tyrosine produced following inflammation are increased in aging skin (13). Specifically, exposure to ultraviolet B promotes the production of tumor necrosis factor (TNF)-α from dermal fibroblasts, macrophages and epidermal keratinocytes, resulting in increased inflammation and the degradation of ECM components via the activation of matrix

metalloproteinases (MMPs) (14-18). Sustained exposure to TNF-α induces the expression of MMP-1, MMP-3 and MMP-9 (also known as collagenase, stromelysin and gelatinase, respectively), causing irreparable damage to the epidermis and dermis (19-21). In particular, MMP-1 activation in dermal fibroblasts may cause collagen fragmentation and functional alterations in dermal fibroblasts (22). Thus, regulating TNF-α activity may be a novel therapeutic strategy of treating inflammatory skin diseases and reversing skin aging.

Type I collagen, the primary component of ECM in the skin, is synthesized and secreted by dermal fibroblasts. Type I collagen is responsible for the strength and elasticity of skin (23). In addition to aging, the stimulation of various cytoplasmic signal transduction pathways, including transforming growth factor (TGF)-β/Smad, mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB, cause fibroblasts to lose their proliferative potential. Fibroblasts then synthesize reduced levels of ECM, meaning that the secretion of type I collagen is also reduced (24-28). Impairments in dermal fibroblast functions during aging contribute to skin thinning, as they cause reductions in the quantity of collagen in aged human skin (29). Previous studies have demonstrated that various MAPKs, including extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38, are associated with skin aging (26,30-32). The phosphorylation of ERK 1/2 mediates the inhibition of type I collagen synthesis in human skin fibroblasts (32,33). TNF-α increases MMP-1 expression via MAPK and activator protein-1 (AP-1) pathways in rheumatoid arthritis synovial fibroblasts (34) and HCS-2/8 chondrocytes (35). Furthermore, it has been demonstrated that compound K suppresses ERK activation, resulting in reduced MMP-1 secretion and increased type I procollagen secretion in TNF-α-stimulated human skin fibroblasts, suggesting that compound K acts as an anti-aging agent (36). However, to the best of our knowledge, there have been no previous studies assessing the ability of GAGs on attenuating TNF-α-induced type I collagen denaturation or the molecular mechanisms underlying the anti-inflammatory effects in human dermal fibroblasts.

The present study assessed the effect of GAGs on the collagen synthesis process in TNF-α-stimulated human dermal fibroblasts (HDFs). The results demonstrated that the molecular mechanism underlying the inhibitory effect of GAGs is associated with the inhibition of ERK/AP-1 signaling. These data indicate that GAGs may serve a critical role in the attenuation of skin inflammation and aging.

## Materials and methods

*Materials*. Recombinant human TNF-α (cat. no. RC214-12) was purchased from Bio Basic, Inc. (Markham, ON, Canada). PD98059 (cat. no. P215) was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Antibodies against phosphorylated (p)-ERK (cat. no. #9101), ERK (cat. no. #9102), p-p38 (cat. no. #4511), p38 (cat. no. #9212), JNK (cat. no. #9252), p-c-fos (cat. no. #5348), c-fos (cat. no. #2250), p-c-jun (cat. no. #9164), c-jun (cat. no. #9165), total caspase-3 (cat. no. #9662), cleaved caspase-3 (cat. no. #9662) and TIMP-1 (cat. no. #8946) were all purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibody against MMP-1 (cat. no. ab137332) was purchased from Abcam (Cambridge, UK) and the antibodies against Bax (cat. no. sc-7480), Bcl-2 (cat. no. sc-492) and β-actin (cat. no. sc-47778) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The antibody against collagen type I (cat. no. NB600-408) was purchased from Novus Biologicals, LLC (Littleton, CO, USA) and the antibody against p-JNK (cat. no. 612541) was purchased from BD Transduction Laboratories; BD Biosciences (Franklin Lakes, NJ, USA). GAG complexes were provided by Taeyoung cosmetics (Elensilia, Seongnam, Korea).

*Cell cultures and cell viability assay*. HDFs were obtained by skin biopsy from one healthy male donor aged 12 years old on September 2014 (Chung-Ang University Hospital, Seoul, Seoul, Korea). The present study was approved by the Ethical Committee of Chung-Ang University Hospital. Written informed consent was obtained from the legal guardians of the donor prior to enrolment. Primary explant cultures were established in 60-$cm^2$ culture flasks in Dulbecco's modified Eagle's medium (DMEM; Welgene, Inc., Gyeongsan, Korea) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 10 $\mu$g/ml streptomycin. HDFs were maintained at 37˚C in a humidified atmosphere of 95% air and 5% $CO_2$. HDFs at passages 3-8 were used for experiments. To investigate the effect of GAGs, HDFs were pretreated with GAGs (0, 0.1, 0.5, and 1%) for 30 min and were then stimulated with TNF-α (20 ng/ml) for 24 h. Cell viability was determined using an MTT assay following the method described by Twentyman and Luscombe (37), with minor modifications. HDFs were seeded at a density of $5x10^4$ cells/well in 24-well plates. Prior to treatment, cells were cultured for 24 h in serum-free DMEM, which was followed by treatment with GAGs (0, 0.1, 0.5, and 1%) for 24 h. HDFs were then incubated with 5 mg/ml MTT for 4 h prior to addition of dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added to dissolve the formazan crystals. Following MTT assays, absorbance was measured at 570 nm using a SpectraMax i3 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

*Enzyme-linked immunosorbent assay (ELISA)*. ELISA was performed using a procollagen type I C-peptide ELISA assay kit (cat. no. MK101; Takara Bio, Inc., Otsu, Japan) and an MMP-1 ELISA kit (cat. no. DY900-05; R&D Systems, Inc., Minneapolis, MN, USA) following the manufacturer's protocol. HDFs were pretreated with GAGs (0, 0.1, 0.5, and 1%) for 30 min and were then stimulated with TNF-α (20 ng/ml) for 24 h. Other HDFs were pretreated with 20 $\mu$M PD98059 (an ERK inhibitor) for 1 h and were then stimulated with TNF-α (20 ng/ml) for 24 h. Collected supernatants (obtained from conditioned media) were used for ELISA and relative absorbance was measured at 450 nm using the SpectraMax i3 microplate reader.

*Reverse transcription quantitative polymerase chain reaction (RT-qPCR)*. Total RNA was extracted from HDFs using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). First-strand cDNA synthesis from the total RNA template was performed using the PrimeScript™ RT master mix (Takara Bio, Inc.). The reverse transcription product was subsequently diluted with

Table I. Oligonucleotide primers used for quantitative polymerase chain reaction.

| Gene | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|
| GAPDH | CACCCACTCCTCCACCTTTGAC | GTCCACCACCCTGTTGCTGTAG |
| COL1A1 | ATCAACCGGAGGAATTTCCGT | CACCAGGACGACCAGGTTTTC |
| MMP-1 | GATGTGGCTCAGTTTGTCCTCAC | CTTGGCAAATCTGGCGTGTAAT |

CoL1A1, collagen type I α-1; MMP-1, matrix metalloprotease-1.

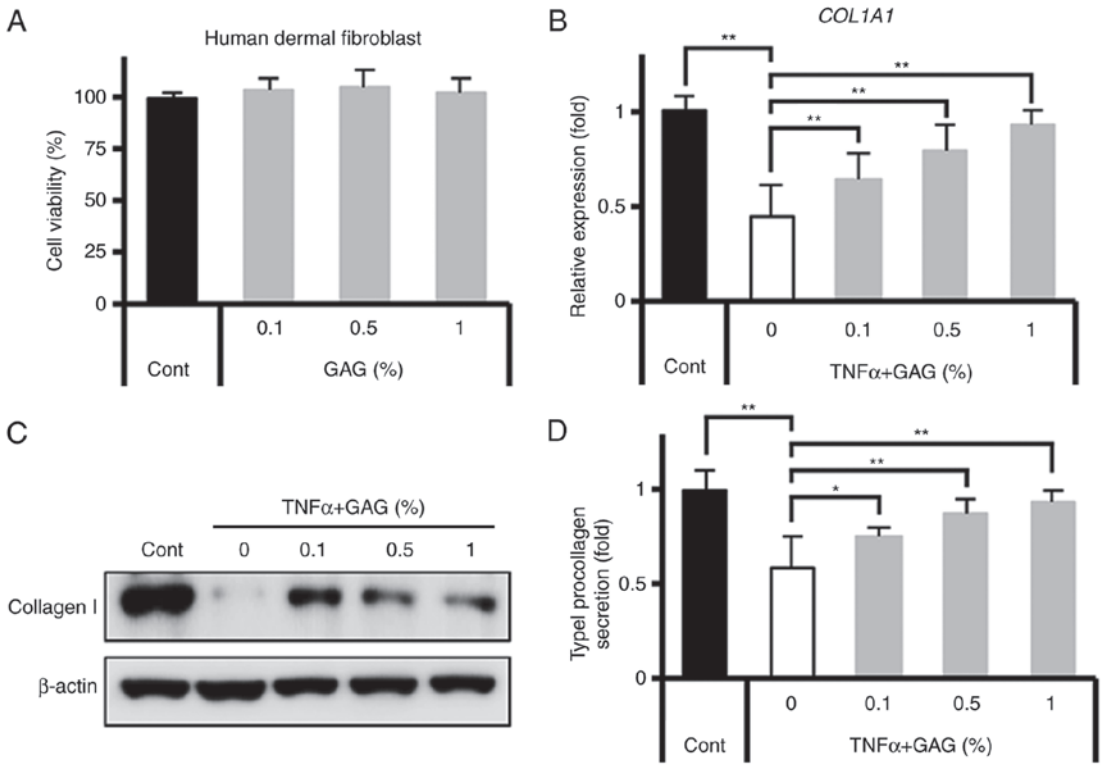


Figure 1. GAGs are required for type I collagen production in TNF-α-stimulated HDFs. HDFs were pretreated with GAGs at the indicated concentrations (0.1, 0.5 and 1%) for 30 min, and then stimulated with TNF-α (20 ng/ml) for 24 h. (A) Cell viability following treatment with GAGs was evaluated using an MTT assay. (B) The expression of collagen I relative to the expression of GAPDH was determined using reverse transcription-quantitative polymerase chain reaction. (C) Lysates from HDFs were immunoblotted with anti-type I collagen and anti-β-actin, antibodies, respectively. (D) Type I procollagen secretion was determined using an ELISA kit. All data are presented as the mean ± standard error of the mean from three independent experiments. $^{*}$P<0.05 and $^{**}$P<0.01. GAGs, glycosaminoglycans; HDF, human dermal fibroblast; TNF-α, tumor necrosis factor-α; Cont, control.

200 $\mu$l $H_2O$. The resulting cDNA was subjected to quantitative PCR using TOPreal™ qPCR 2X PreMIX SYBR (Enzynomics, Daejeon, Korea) with a CFX-96 Touch™ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The thermocycling conditions used to amplify all genes were as follows: 10 min at 95˚C, 40 cycles of 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec. Expression data were calculated from the cycle threshold (Cq) value using the ΔΔCq method of quantification (38). Gene expression values were normalized to the expression of GAPDH. The sequences of the oligonucleotide primers used for qPCR are presented in Table I.

*Cell lysate preparation and western blot analysis*. Cell extracts were prepared as described previously (39). Treated whole cell extracts were lysed in radioimmunoprecipitation buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate] containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Protein concentrations were determined using a BCA assay kit (Thermo Fisher Scientific Inc.). For western blot analysis, cell lysates, each containing 20-30 $\mu$g protein, were resolved using 8-12% SDS-PAGE and were then transferred to polyvinylidene fluoride membranes. Membranes were soaked in 5% skim milk blocking buffer for 1 h at room temperature. Subsequently, membranes were incubated with anti-collagen I (1:1,000), anti-MMP-1 (1:2,500), anti-TIMP-1 (1:2,500), anti-p-ERK (1:2,500), anti-ERK (1:2,500), anti-p-p38 (1:2,500), anti-p38 (1:2,500), anti-p-JNK (1:2,500), anti-JNK (1:2,500), anti-p-c-Fos (1:2,500), anti-c-Fos (1:2,500), anti-p-c-Jun (1:2,500), anti-c-Jun (1:2,500), anti-Bax (1:1,000), anti-Bcl-2 (1:1,000), anti-total caspase-3 (1:2,500), anti-cleaved caspase-3

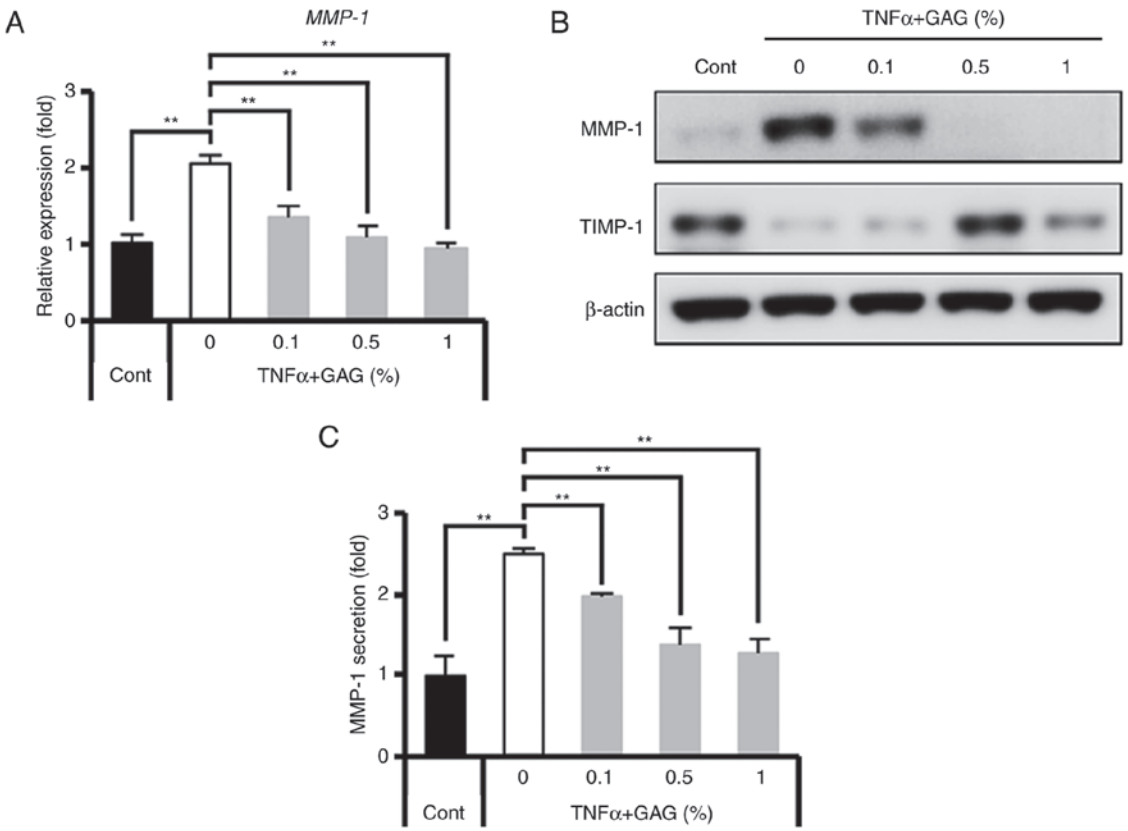


Figure 2. GAGs are required for the inhibition of MMP-1 expression in TNF-α-stimulated HDFs. HDFs were pretreated with GAGs at the indicated concentrations (0.1, 0.5 and 1%) for 30 min and then stimulated with TNF-α (20 ng/ml) for 24 h. (A) The expression of MMP-1 relative to the expression of GAPDH was determined using reverse transcription-quantitative polymerase chain reaction. (B) Lysates from HDFs were immunoblotted with anti-MMP-1, anti-TIMP-1 and anti-β-actin, respectively. (C) MMP-1 secretion was determined using an ELISA kit. All data are presented as the mean ± standard error of the mean from three independent experiments. $^{**}$P<0.01. GAGs, glycosaminoglycans; MMP-1, matrix metalloproteinase-1; TNF-α, tumor necrosis factor-α; HDF, human dermal fibroblast; TIMP-1, tissue inhibitor of metalloproteinase-1; Cont, control.

(1:2,500) and anti-β-actin (1:1,000; loading control) antibodies overnight at 4˚C. Then, the membrane was incubated with anti-mouse (cat. no. PI-2000, 1:10,000), anti-rabbit (cat. no. PI-1000, 1:10,000; Vector Labs Inc., Burlingame, CA, USA) secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature. Membranes were developed using enhanced chemiluminescence western blot detection reagents (GE Healthcare, Chicago, IL, USA). Immunoblots were analyzed using ImageJ 1.44 software (National Institutes of Health, Bethesda, MD, USA).

*Statistical analyses*. All quantitative data are presented as the mean ± standard error of the mean for three independent experiments. Statistical analyses were performed using the statistical package for SPSS software version 10.0 (SPSS, Inc., Chicago, IL, USA). Differences between the two groups were evaluated using a paired t-test. For multiple comparisons, one-way analysis of variance was used followed by Tukey's multiple comparisons test. P<0.05 was considered to indicate a statistically significant result.

## Results

*GAGs regulate type I collagen production in TNF-α-stimulated human dermal fibroblasts*. Firstly, to measure the cytotoxic effect of GAGs, HDFs were serially treated for 24 h with various concentrations of GAGs (0, 0.1, 0.5 and 1%). The results demonstrated that GAGs had no significant effect on cell viability at any of the tested concentrations (Fig. 1A). To identify the protective effect of GAGs against factors that influence skin aging, the effect of GAGs on TNF-α-induced type I collagen production in HDFs were assessed. HDFs were pretreated with GAGs for 30 min and were then stimulated with TNF-α (20 ng/ml) for 24 h. The expression of type I collagen decreased significantly following stimulation with TNF-α (Fig. 1B). However, treatment with GAGs significantly reversed this decrease in type I collagen expression in a dose-dependent manner (Fig. 1B and C). Furthermore, type I collagen secretion by HDFs following stimulation was significantly decreased; this decrease was reversed following treatment with GAGs (Fig. 1D). These results suggest that GAGs regulate the production of type I collagen in TNF-α-stimulated HDFs.

*GAGs enhance type I collagen production via MMP-1 inhibition*. The results demonstrated that GAGs reversed the reduction in type I collagen levels that occurred in TNF-α-stimulated HDFs (Fig. 1). The regulatory proteins of collagen degradation were further assessed as many enzymes, including MMPs and tissue inhibitors of metalloproteinase (TIMPs), are directly and indirectly involved in collagen

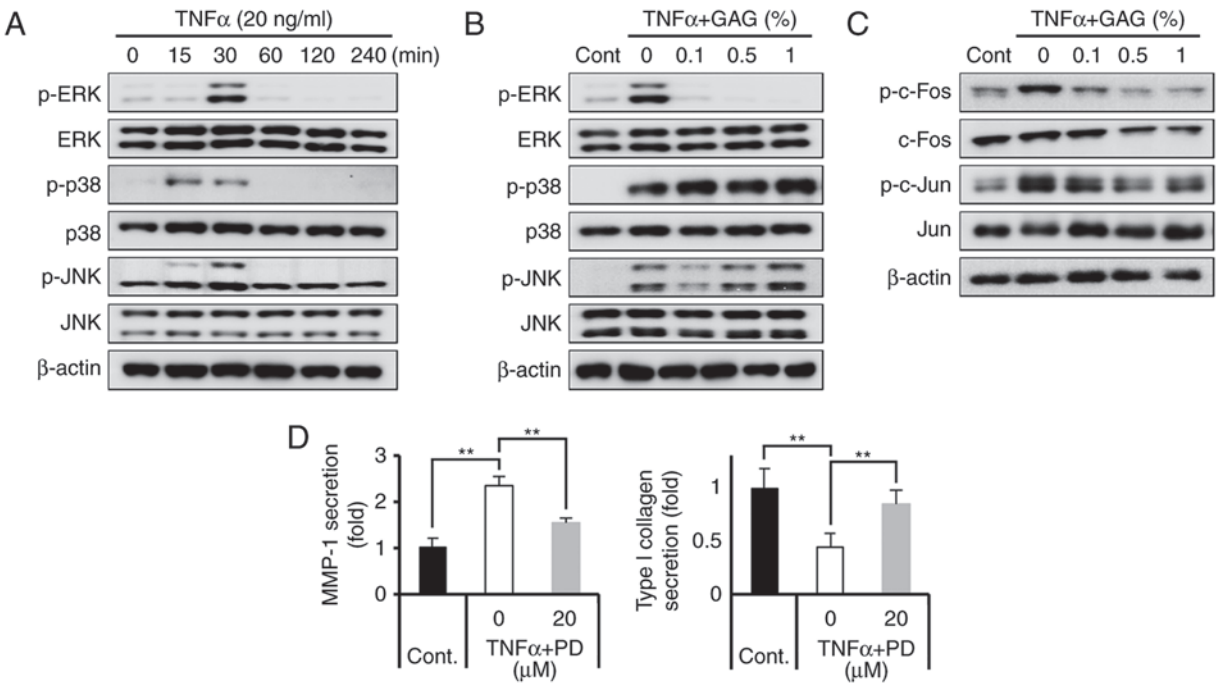


Figure 3. GAGs regulate type I collagen degradation by inhibiting MMP-1 via the ERK/AP-1 cascade in TNF-α-stimulated HDFs. (A) HDFs were treated with TNF-α (20 ng/ml) for 0, 15, 30, 60, 120 and 240 min, and lysates from HDFs were immunoblotted with anti-p-ERK, anti-ERK, anti-p-p38, anti-p38, anti-p-JNK, anti-JNK and anti-β-actin antibodies. (B) HDFs were pretreated with GAGs at the indicated concentrations (0, 0.1, 0.5 and 1%) for 30 min and then stimulated with TNF-α (20 ng/ml) for 30 min. Lysates from HDFs were immunoblotted with anti-p-ERK, anti-ERK, anti-p-p38, anti-p38, anti-p-JNK, anti-JNK and anti-β-actin antibodies. (C) Lysates from HDFs were immunoblotted with anti-p-c-fos, anti-fos, anti-p-c-jun, anti-c-jun and anti-β-actin antibodies. (D) HDFs were pretreated with 20 $\mu$M PD98059 for 1 h and then stimulated with TNF-α (20 ng/ml) for 24 h. MMP-1 secretion and type I collagen secretion were subsequently determined using an ELISA kit. All data are presented as the mean ± standard error of the mean for three independent experiments. $^{**}$P<0.01. GAGs, glycosaminoglycans; MMP-1, matrix metalloproteinase-1; ERK, extracellular signal-regulated kinase; TNF-α, tumor necrosis factor-α; HDF, human dermal fibroblast; p-, phosphorylated; JNK, c-jun N-terminal kinase; Cont, control; PD, PD98059.

type I degradation (40-43). The results demonstrated that GAGs inhibited MMP-1 gene activation (Fig. 2A and B) and also MMP-1 secretion (Fig. 2C) in TNF-α-stimulated HDFs in a dose-dependent manner. However, the expression of TIMP-1, a tissue inhibitor of metalloproteinases, decreased in TNF-α-stimulated HDFs, but increased following GAG treatment, peaking following treatment with 0.5% GAGs (Fig. 2B). Subsequently, the molecular mechanism by which MMP-1 production is inhibited in HDFs by GAGs following stimulation with TNF-α was assessed.

*GAGs inhibit MMP-1 expression via suppression of the ERK signaling pathway*. Previous studies have indicated that MAPK pathways, specifically those induced by TNF-α, serve an important role in the regulation of procollagen synthesis (26,30-32,35,44). Thus, the present study assessed TNF-α induced MAPK signaling. The results demonstrated that TNF-α activated the phosphorylation of ERK, JNK and p38, peaking at 30 min and then the signal gradually weakened (Fig. 3A). To determine whether GAGs suppressed the activation of MAPK, HDFs were pretreated for 30 min with GAGs (0, 0.1, 0.5 and 1%) and then stimulated with TNF-α for a further 30 min. The results demonstrated that GAGs attenuated ERK phosphorylation but did not affect the phosphorylation of JNK and p38 (Fig. 3B). In addition, GAGs markedly attenuated the phosphorylation of AP-1, including c-fos and c-jun, the major transcription factors downstream of ERK that are responsible for the expression of MMP-1 (Fig. 3C). The results confirmed that MMP-1 activation and type I collagen regulation is dependent on ERK signaling using the MEK/ERK inhibitor PD98059 in HDFs. Treatment with PD98059 (20 $\mu$M) significantly attenuated the upregulation of MMP-1 by TNF-α (Fig. 3D). Furthermore, treatment with PD98059 resulted in recovered type I collagen secretion (Fig. 3D). These results confirmed that GAGs inhibit MMP-1 expression via ERK inactivation, followed by an increase in type I collagen production.

*GAGs inhibit the TNF-α-induced-apoptosis of HDFs*. TNF-α is a pleiotropic cytokine with diverse cellular responses. TNF-α induces apoptosis in different types of cells, including fibroblasts (45-47). Therefore, the present study assessed whether GAGs inhibit TNF-α-induced HDF apoptosis. HDFs were pretreated with GAGs for 30 min and then stimulated with TNF-α for 24 h. Cell apoptosis was then evaluated by assessing the expression of the apoptosis-associated proteins caspase-3, Bcl-2-associated X protein (Bax) and B-cell lymphoma 2 (Bcl-2). Caspase-3 serves a key role in TNF-α-mediated apoptosis (48,49). The results demonstrated that TNF-α stimulation markedly increased cleaved caspase-3 levels (as indicated by the bottom band on the western blot in Fig. 4A) and pro-apoptotic Bax levels. However, treatment with GAGs markedly abolished these increases (Fig. 4A). Additionally, GAGs treatment did not alter levels of the anti-apoptotic protein Bcl-2, but restored the Bax/Bcl-2 ratio following stimulation with TNF-α in HDFs, in a dose-dependent manner (Fig. 4A and B). These

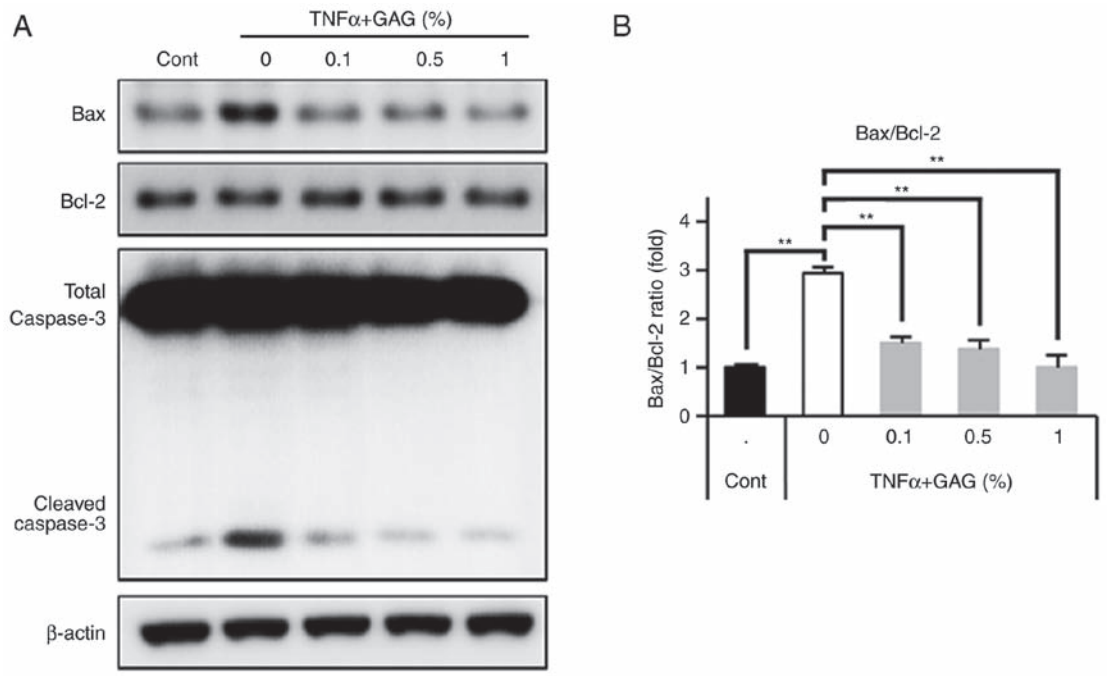


Figure 4. Anti-apoptotic effect of GAGs in TNF-α-stimulated HDFs. HDFs were pretreated with GAGs at the indicated concentrations (0, 0.1, 0.5 and 1%) for 30 min and then stimulated with TNF-α (20 ng/ml) for 24 h. (A) Lysates from HDFs were immunoblotted with anti-Bax, anti-Bcl-2, anti-caspase-3 and anti-β-actin antibodies. (B) Western blots were analyzed quantitatively. All data are presented as the mean ± standard error of the mean for three independent experiments. **P<0.01. GAGs, glycosaminoglycans; TNF-α, tumor necrosis factor-α; HDF, human dermal fibroblast; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; Cont, control.

results indicate that GAGs protect HDFs against the apoptosis induced by TNF-α.

## Discussion

The various anti-aging benefits of GAGs have contributed to their widespread inclusion in cosmetic and pharmaceutical products (50,51). GAGs, including hyaluronic acid, chondroitin sulfate, dermatan sulfate and keratan sulfate are abundant structural components of extracellular structures and regulate many biological processes, including cell growth, migration, differentiation, adhesion and lipid synthesis in the dermis and epidermis (1,52-56). The contents of GAGs change during intrinsic and extrinsic aging (57-60). Specifically, GAGs containing proteoglycans, including versican, decorin and biglycan, serve important roles in the synthesis and maintenance of collagen and elastin (61-64). It has been demonstrated that the removal of collagen-attached GAGs affects collagenolysis via cathepsin K (65). However, few studies have reported the molecular mechanisms of GAGs underlying the anti-aging effects on the skin.

To the best of our knowledge, the present study is the first to demonstrate that GAGs significantly inhibit TNF-α-induced type I collagen denaturation in HDFs. It has been demonstrated that versican, a member of the chondroitin sulfate proteoglycan (PG) family, binds to collagens and controls fibril formation (64). In addition, deficiencies in decorin and biglycan, which are members of the dermatan sulfate PG family, cause abnormal collagen fibril formation and result in thin and fragile skin (61-63). The keratan sulfate PGs, lumican and fibromodulin, are also able to regulate the formation of collagen fibrils (66).

The molecular components of the ECM are remodeled by matrix metalloproteinases released from fibroblasts (67). Collagen degradation can be induced by MMP-1 and the expression and activity of MMP-1 may become elevated as a result of intrinsic and extrinsic aging (68,69). The present study demonstrated that GAGs inhibit the TNF-α-induced increase in MMP-1 expression in HDFs. However, the results demonstrated that levels of TIMP-1, an inhibitor of MMP-1, increased along with the concentration of GAGs in HDFs stimulated with TNF-α. The results of the present study therefore support the conclusion that MMPs and TIMPs are directly and indirectly involved in the synthesis of collagen type I (40-43).

The present study also assessed the upstream signal transduction pathways of collagen synthesis via the regulation of MMP-1 and TIMP-1. Previous studies have demonstrated that MAPK signaling pathways serve pivotal roles in controlling various cellular functions, including cell growth, MMP expression and collagen synthesis (26,30-32). In addition, several studies have revealed that MMP-1 expression is enhanced by activated MAPK in dermal fibroblasts following stimulation with particular stimuli (70,71). The present study demonstrated that GAG treatment resulted in the suppression of ERK phosphorylation (but not the phosphorylation of JNK or p38) in TNF-α-induced HDFs, leading to the reduced phosphorylation of AP-1. It has also demonstrated that tensile force induces the expression of type I collagen and MMP-1, and activates MAPKs in periodontal ligament fibroblasts (72). In particular, the inhibition of ERK, but not JNK or p38 MAPK, negatively regulates the tensile force-mediated activation of NF-κB and MMP-1 expression (72). The

TNF-α-induced expression of MMP-9 was increased via the Ras/ERK-regulated activation of NF-κB and AP-1 in human vascular smooth muscle cells (73). In addition, various cytokines, including interleukin (IL)-1β, IL-6, IL-8 and inducible nitric oxide synthase induced by MAPKs, are involved in the inflammation associated with skin diseases (74,75). Thus, further studies are required to assess the anti-inflammatory effect of GAGs in skin diseases or in conditions associated with aging.

Finally, the current study confirmed the anti-apoptotic effects of GAGs in HDF stimulated with TNF-α. It has established that TNF-α stimulates a variety of responses, including inflammation and apoptosis *in vitro* and *in vivo* (76-78). The present study demonstrated that GAGs exert anti-apoptotic effects in HDFs stimulated with TNF-α. Furthermore, a previous study determined that TNF-α-induced ERK activation mediates p53 activation in apoptotic and autophagic L929 cells (79). In addition, PD98059 has been demonstrated to significantly reduced the cytotoxic effect of TNF-α in L929 and U251 cells (80).

In conclusion, the results of the current study indicate that GAGs serve an important role in type I collagen production. Specifically, GAGs reduced MMP-1 expression and elevated TIMP-1 expression by inhibiting the ERK/AP-1 cascade in TNF-α-stimulated HDFs. In addition, a protective effect of GAGs against cell apoptosis was observed. A previous study demonstrated that a cream comprised of GAGs positively regulated wrinkles, skin elasticity, dermal density and skin tightening in a clinical trial (4). Given that the expression of various proinflammatory cytokine/chemokines are significantly elevated and the expression of type I collagens is decreased in aged skin dermis, the current study suggests that GAGs may be used as anti-inflammatory and anti-aging agents for skin.

**Acknowledgements**

Not applicable.

**Funding**

The present study was supported by Elensilia, Taeyoung Co., Korea (grant no. 20130994).

**Availability of data and materials**

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

**Authors' contributions**

BJK, YL, and JN designed the research. JN, DHB, and SII conducted the research. BJK, YL, JN, DHB, HC, JHH, SYK, and YAN analyzed the data. All authors read and approved the final manuscript.

**Ethical approval and consent to participate**

The Ethical committee of Chung-Ang University Hospital Institute Review Board [IRB no. C2015051 (1509)] approved the present study. Written informed consent was obtained from the legal guardians of the donor prior to enrolment.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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