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Research Note

Synergistic effect of maclurin on ginsenoside compound K induced inhibition of the transcriptional expression of matrix metalloproteinase-1 in HaCaT human keratinocyte cells

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Matrix metalloproteinases (MMPs) are enzymes that can degrade various proteins comprising the extracellular matrix (ECM) [1]. They are well known for their close relationship with cancer metastasis and skin aging [2]. More than 20 MMPs have been reported so far, and these include major gelatinases (MMP-2 and MMP-9) and collagenases (MMP-1, MMP-8, and MMP-13) [2–4]. The collagenases have the very specialized ability to destroy the collagen triple helix. As a result, collagen chains are unwound to be further destroyed by other MMPs. Particularly, MMP-1 is the most abundant of these collagenases and breaks down collagen types 1, 2, and 3 [1]. Among the various protein components comprising the ECM, collagen is the most abundant [5]. The ECM functions as a physical and biochemical barrier for cells migrating from their original site and maintains the structural integrity of the skin dermis [6]. Therefore, degradation of collagen is a critical step in cancer cell metastasis and a major cause of skin aging.

In Northeast Asian countries including Korea, China, and Japan, Korean ginseng has been regarded as a valuable herbal medicine. Ginsenosides are the major active components exerting pharmacological characteristics of ginseng. Of the ginsenosides known so far, Rb1 ginsenoside from *Panax ginseng* is the most abundant one and the source of ginsenoside compound K [7]. Compound K (CK), 20-O-b-D-glucolyranosyl-20(S)-protopanaxadiol, was first isolated from soil bacteria [7–11] (Fig. 1A). Among the many biological functions investigated so far, the skin protective effects of CK through the inhibition of MMP-1 has drawn attention of researchers [10,12,13].

Maclurin [(3,4-dihydroxyphenyl)-(2,4,6-trihydroxyphenyl) methanone] is a natural compound belonging to the benzophenone family and is ethanol-extracted from *Morus alba* and *Garcinia mangostana* (Fig. 1B). Maclurin was reported as one of the five major phenolic components of the ethanol extract of mulberry twigs (resveratrol, rutin, morin, isoquercitrin, and maclurin) and found to possess antioxidative activity via the inhibition/reduction of superoxide [14]. It was reported earlier that maclurin has antimetastatic effects in human non–small cell lung cancer cells via inhibitions of two major gelatinases, MMP-2 and MMP-9 [15]. The inhibitory effect on these two metalloproteinases was closely related to the suppression of the transcriptional expression of both MMP-2 and MMP-9. Based on this phenomenon, maclurin was suggested to be developed as a potential antimetastatic agent for various tissue-specific human cancers. In this study, I decided to link the suppressive effect of maclurin on MMPs to the development of functional agents for the prevention of skin aging. Because mulberry twigs are agricultural waste, maclurin would be a relatively inexpensive and environment-friendly material to be used in combination with relatively costly CK. To test the possibility to be used as synergistic material on CK-induced MMP-1 inhibition, the inhibitory effect of maclurin on MMP-1 was investigated in HaCaT human keratinocyte cells. Because collagens are the most abundant proteins comprising the ECM, which gets broken down during skin aging, inhibition of the collagen-degrading MMP-1 enzyme may be critical for the prevention of skin aging. To study the effect of maclurin on MMP-1 activity in HaCaT human keratinocyte cells, a collagen zymography assay was performed. HaCaT human keratinocyte cells were maintained in Dulbecco's Modified Eagle's Media

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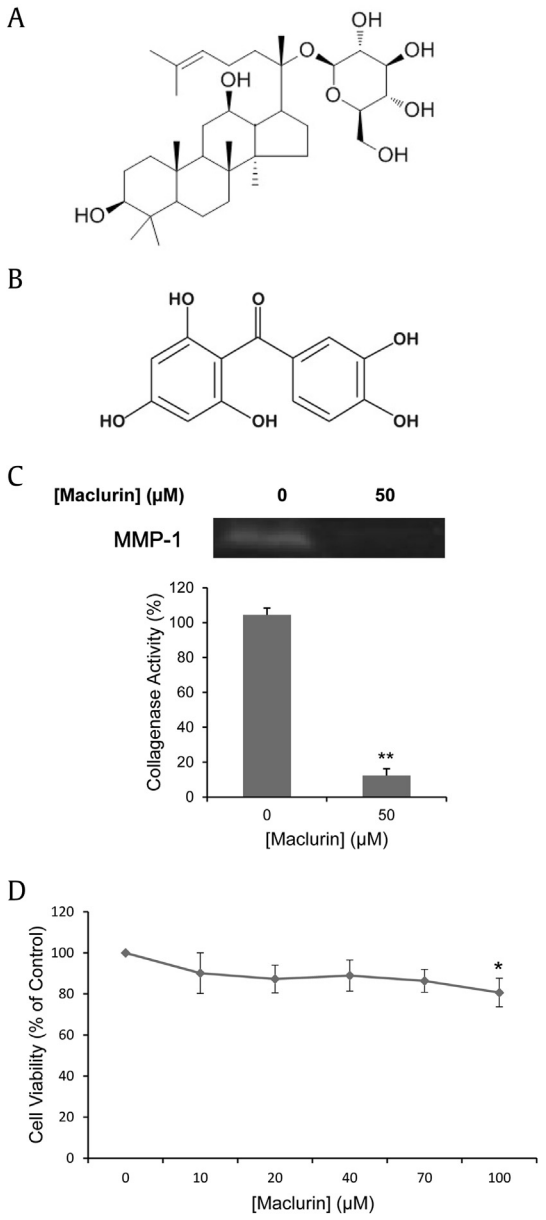

Fig. 1. Inhibitory effect of maclurin on collagenase activity in HaCaT human keratinocyte cells. (A) Chemical structure of CK. (B) Chemical structure of maclurin. (C) Collagenase activity of MMP-1 was inhibited by maclurin in HaCaT human keratinocyte cells. (D) Cytotoxicity of maclurin was tested. Cell viability was assayed using the CCK-8 Kit. The results were statistically evaluated using Student *t* test. $^{*}p < 0.05$; $^{**}p < 0.01$. CCK-8, Cell Counting Kit-8; CK, compound K; MMP, matrix metalloproteinase.

(DMEM) (HyClone Laboratories, Inc, South Logan, UT, USA) supplemented with 10% fetal bovine serum and 100 U/mL of penicillin and 100 mg/mL streptomycin mixed antibiotics. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (with 0.1% collagen) was performed, and gels were washed with zymography washing buffer (2.5% Triton X-100 in distilled water). Then, the gels were incubated in zymography development solution (0.5 M Tris-HCl, pH 7.6, 5 mM $CaCl_2$) for 4 h at 37°C. The gels were stained and destained with Coomassie Brilliant Blue R staining solution and destaining solution (20% methanol, 10% acetic acid, and 70% distilled H_2O). The unstained bands on the gel indicate collagenase activity. As shown in Fig. 1C, the collagenase activity of MMP-1 was inhibited by maclurin, indicating the MMP-1 suppressive effect of maclurin. A possible cytotoxic effect of a compound may limit the opportunity for it to be further developed as a functional additive for antiaging skin products. To investigate the cytotoxic effect of maclurin on HaCaT human keratinocyte cells, a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc., Rockville, MD, USA) assay was performed according to the manufacturer's instructions. The cells were seeded at a density of 10^4 cells/well in 96-well plates with 10% fetal bovine serum and incubated overnight. Cells were exposed to increasing concentrations of maclurin for 24 h, and cell viability was subsequently measured. As shown in Fig. 1D, the cytotoxicity of maclurin was not significant as indicated by the cell viability at any dosage of maclurin. This result suggests that maclurin is not significantly toxic to HaCaT human keratinocyte cells and may allow for this natural material to be further modified and developed as a candidate functional agent for antiaging cosmetic products.

To determine whether the downregulation occurs at the transcriptional level, quantitative reverse transcription–polymerase chain reaction for MMP-1 was performed. RNA samples from experimental cells were extracted using the RNeasy Kit (Qiagen, Hilden, Germany). Reverse transcription for the synthesis of cDNA was executed using a cDNA Synthesis Kit (PhileKorea Technology, Inc, Daejeon, Korea). The QuantiSpeed SYBR Kit (PhileKorea) was used for quantitative real-time polymerase chain reaction. Primer sequences for hMMP-1 are 5′-GAG ATCATCGGGACAACTCTCCTT-3′ (forward) and 5′-GTTGGTCCACCTTTCATCTTCAT CA-3′ (reverse). Forty sequential cycles of denaturation (5 s at 95°C), annealing, and

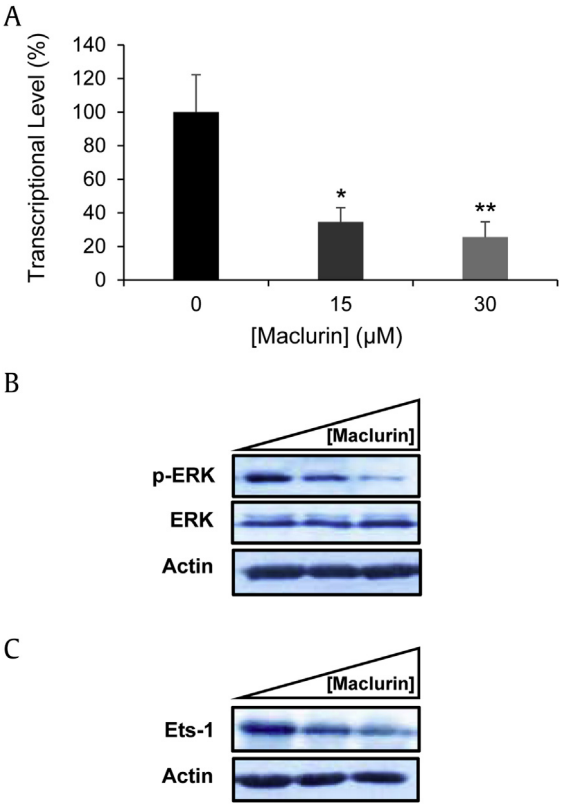

Fig. 2. Maclurin inhibits the transcriptional expression of MMP-1 and ERK/Ets-1 signaling in HaCaT human keratinocyte cells. (A) The mRNA level of MMP-1 was lowered by maclurin treatment (0, 15, and 30 μM) in HaCaT human keratinocyte cells. The result was statistically evaluated using Student *t* test. $^{*}p < 0.05$; $^{**}p < 0.01$. (B) Effect of maclurin on ERK was investigated by Western blot analysis. Activated ERK (represented by p-ERK) was decreased proportionally to the concentrations of applied maclurin (0, 50, and 100 μM). (C) Cellular level of Ets-1 was downregulated by maclurin in HaCaT human keratinocyte cells in a dosage-dependent manner. MMP, matrix metalloproteinase.

extension (15 s at 60℃) were performed. Fluorescence of SYBR Green was monitored by the Rotor-Gene Q (Qiagen) for the determination of Ct values. The $2^{-\Delta\Delta Cp}$ method was used for the analysis of relative gene expression. As seen in Fig. 2B, the level of MMP-1 mRNA was significantly attenuated by maclurin treatment of HaCaT human keratinocyte cells. This may strongly indicate that the suppressive effect of maclurin on MMP-1 in HaCaT cells is the consequence of the downregulation of the transcriptional expression by maclurin. These results indicate that maclurin may be worth developing as a functional agent for the prevention of skin aging. Among the various cellular signaling pathways, mitogen-activated protein kinases and AKT signaling are well known to be related with MMP expressions [2,16]. Therefore, the effect of maclurin on activation of three representative mitogen-activated protein kinases, Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, was investigated by Western blot analysis. As seen in Fig. 2B, the activation of ERK (represented by p-ERK) was significantly downregulated by maclurin in a dosage-dependent manner. The activation of AKT was not suppressed by maclurin as indicated by the Western blot bands of p-AKT, which is an active form of AKT (data not shown here). It was reported that the Activator protein 1 (AP-1) family member c-Jun and the Ets-1 transcription factors are phosphorylated and activated by ERK [17,18]. In addition, Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) transcription factor is well known as a major regulating factor for MMP-1 expression [19]. Among the three factors, Ets-1 was downregulated by maclurin (Fig. 2C). These results indicate that maclurin exerts its inhibitory effect on the transcriptional expression of MMP-1 via ERK/Ets-1 signaling.

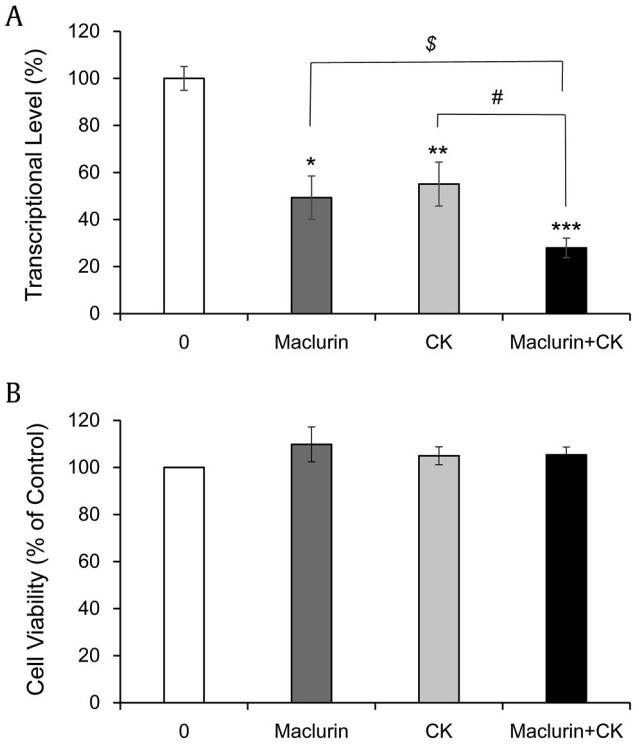

Fig. 3. Synergistic effect of maclurin on CK-induced inhibition of the transcriptional expression of MMP-1 in HaCaT human keratinocyte cells. (A) The mRNA level of MMP-1 was more lowered by the combinatorial treatment of CK and maclurin (15 μM and 5 μM, respectively) than by the sole treatment of maclurin (15 μM) and CK (5 μM). (B) Cytotoxicity of the combination of CK and maclurin was tested in HaCaT human keratinocyte cells. Cells were exposed to 15 μM of maclurin, 5 μM of CK, and both maclurin/CK (15 μM and 5 μM, respectively) for 24 h. Cell viability was assayed using the CCK-8 Kit. The results were statistically evaluated using Student *t* test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$;$\#p < 0.05$; $\$p < 0.05$). CCK-8, Cell Counting Kit-8; CK, compound K; MMP, matrix metalloproteinase.

After the verification of maclurin as a potential molecule possibly used to inhibit MMP-1 together with CK, the synergistic effect of maclurin on CK-induced inhibition of the transcription of MMP-1 was evaluated by quantitative reverse transcription–polymerase chain reaction (Fig. 3A). The level of MMP-1 mRNA was significantly more downregulated when the combination of CK and maclurin was treated (15 μM of maclurin and 5 μM of CK) than when maclurin or CK was solely treated (15 μM and 5 μM, respectively).

To test a possible cytotoxic effect of the combination of CK and maclurin, CCK-8 cell viability assay was performed (Fig. 3B). As seen in Fig. 3B, the cytotoxic effect of the mixture of CK and maclurin was not significant in HaCaT human keratinocyte cells. This may open the chance for this combinatorial application to be further developed as antiaging skin products.

In this study, we found that maclurin downregulates MMP-1 expression in HaCaT human keratinocyte cells and induces synergistic effect on ginsenoside CK-induced inhibition of the transcriptional expression of MMP-1. The results presented here strongly suggest that the combination of CK and maclurin be an effective agent for skin antiaging therapies and, therefore, may have industrial and medicinal value for application in the cosmetic industry and the dermatologic sector.

Conflicts of interest

The author declares no conflicts of interest.

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