Involvement of Catechols in Acteoside in the Activation of Promatrix Metalloproteinase-2 and Membrane Type-1-Matrix Metalloproteinase Expression via a Phosphatidylinositol-3-Kinase Pathway in Human Dermal Fibroblasts

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Granulation tissue formation during skin wound healing requires the migration and proliferation of dermal fibroblasts in the wound site, where a subsequent remodeling of extracellular matrices (ECM) occurs. An abnormality of ECM remodeling within the healing wound leads to fibrosis and a contracted scar. To evaluate whether acteoside, a phenylethanoid glycoside isolated from the leaves of Rehmannia glutinosa (L) BOSCH., exhibits wound-healing actions, we examined the effect of acteoside on the expression of matrix metalloproteinases (MMPs) in normal human dermal fibroblasts (NHDF). Acteoside dose- and time-dependently augmented the activation of the precursor of MMP-2 (proMMP-2/progelatinase A) in untreated- and interleukin-1β-treated NHDF, while the alteration of the MMP-2 gene expression was negligible. The acteoside-induced proMMP-2 activation was associated with the augmented membrane-type 1 MMP (MT1-MMP) expression in the NHDF. In addition, the proMMP-2 activation was enhanced by two aglycons in acteoside: caffeic acid and 3,4-dihydroxyphenylethanol, which consist of catechol. However, there was no change in the proMMP-2 activation in other catechol derivatives: homovanillyl alcohol- and homovanillic acid-treated NHDF, indicating that catechols in acteoside were requisite for the regulation of the MMP activation and expression in NHDF. Furthermore, the proMMP-2 activation by acteoside was selectively inhibited by LY294002, a potent phosphatidylinositol-3-kinase (PI3K) inhibitor. These results provide novel evidence that acteoside augments proMMP-2 activation along with an increase in MT1-MMP expression through a PI3K signal pathway in NHDF. Thus, acteoside is likely to be an attractive candidate that facilitates ECM remodeling in the skin wound repair process.

Key words acteoside; dermal fibroblast; matrix metalloproteinase; granulation tissue formation; extracellular matrix

Cutaneous wound healing is a dynamic and complex process that involves inflammation, granulation tissue formation, and tissue remodeling.1,2 Since inflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor a (TNF-α) have been shown to accelerate wound healing, their excessive expression has caused aberrations in wound healing such as hypertrophic scars, keloids, and ulcers.3,4 Furthermore, a lot of cell species have coordinately and temporally organized the biological reaction to heal a wound, fibroblasts are considered to be important cells for granulation tissue formation and the reconstitution of dermal ECM in wound repair.1

Matrix metalloproteinases (MMPs) are the enzymes that degrade various ECM components in skin under physiological and pathological conditions. The expression and activation of MMPs have been reported to spatially and temporally increase at the early phases of wound healing.5 Among the MMP family, MMP-2 (gelatinase A) has been reported to be involved in cell migration and proliferation during wound healing.6 MMP-2 has been synthesized and secreted as the latent form (proMMP-2), and then activated by membrane type 1-MMP (MT1-MMP) on the cell surface.7 The enzymatic activity of MMP has been reported to be stoichiometrically regulated by tissue inhibitors of metalloproteinases (TIMPs),8 indicating that the regulation of ECM breakdown is necessary for pathophysiological tissue repair.

Herbal medicines have been reported to exhibit various pharmacological effects, including the regulation of ECM remodeling.9 A phenylethanoid glycoside, acteoside (Fig. 1) is widely distributed in various medicinal plants including Rehmannia glutinosa (R. glutinosa) L. Acteoside possesses two kinds of aglycon: caffeic acid (CA) and 3,4-dihydroxyphenylethanol (hydroxytyrosol) (DOPE) (Figs. 1 and S1), both of which consist of catechol, and two types of sugar moieties: glucose and rhamnose.10 Acteoside has been shown to have various biological activities such as anti-inflammatory, antitumor, and neuronal protective activities in vitro and in vivo.9–12 In addition, it has been reported that acteoside interferes with progressive glomerulonephritis through the suppression of the leukocyte accumulation in the glomeruli in rats.13 Moreover, CA and its derivative, caffeic acid phenylester, have been shown to accelerate cutaneous wound healing.14,15 However, it remains unclear whether acteoside modulates the
wound healing activity.

In this study, we investigated the effects of acteoside on the expression and activation of proMMP-2 in normal human dermal fibroblasts (NHDF), and demonstrated that acteoside augmented proMMP-2 activation and MT1-MMP expression. In addition, we found that two catechol derivatives, that are components of acteoside, mimicked the proMMP-2 activation. Furthermore, the acteoside-induced proMMP-2 activation was suppressed by a potent inhibitor of phosphatidylinositol-3-kinase (PI3K) in NHDF. These results suggest that acteoside is an attractive candidate with a wound healing effect due to the enhancement of proMMP-2 activation in NHDF.

MATERIALS AND METHODS

Cell Culture NHDF were purchased from Lonza Walkersville, Inc. (Walkersville, MD, U.S.A.). NHDF were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen Co., Carlsbad, CA, U.S.A.) containing 10% fetal bovine serum (FBS) (Thermo ELECTRON Co., Melbourne, Australia) with antibiotics [100 units/mL of penicillin G (MP Biomedicals Inc., OH, U.S.A.) and 100 µg/mL of streptomycin sulfate (Meiji Seika Ltd., Tokyo, Japan)] until confluence. NHDF at up to the 18th passage were used without any changes in morphology and proliferation rate. NHDF were treated for 4–24 h with acteoside (6.25 to 100 µM) (Fig. 1), which was isolated from the dry leaves of *R. glutinosa* L. (Supplementary materials), in the presence or absence of IL-1β (10 ng/mL), TNF-α (10 ng/mL) (Cosmo Bio Co., Tokyo, Japan), and/or intracellular signaling inhibitors: U0126 (5 and 10 µM), PD98059 (10 and 20 µM), SB203580 (10 and 20 µM), SP600125 (10 and 20 µM), and LY294002 (10 and 20 µM) (Promega Co., Madison, WI, U.S.A.) in DMEM/0.2% lactalbumin hydrolysate (LAH) (Sigma Chemical Co., St. Louis, MO, U.S.A.). Similarly, NHDF were treated for 24 h with concanavalin-A (Wako Pure Chemical Industries, Ltd., Osaka, Japan), CA, DOPA, homovanillyl alcohol (MOPE) or homovanillic acid (HVA) (Sigma Chemical). Acteoside, the intracellular signaling inhibitors, CA, DOPA, MOPE, and HVA were added to the culture medium as a dimethyl sulfoxide (DMSO) solution. The final DMSO concentration was 0.1% in all cultures, and the same vehicle amount was added to the control cultures. The harvested cells and culture medium were stored at −20°C until use.

Gelatin Zymography ProMMP-2 activation was analyzed by gelatin zymography as previously described. Briefly, the culture medium was mixed with a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer without 2-mercaptoethanol, and then subjected to an SDS-PAGE using polyacrylamide gel containing 0.9% gelatin. After electrophoresis, the gel was washed for 1 h with a washing buffer [50 mM Tris–HCl (pH 7.5), 5 mM CaCl₂, 1 mM ZnCl₂, 2.5% Triton X-100] followed by incubating for 7 h in a reaction buffer [50 mM Tris–HCl (pH 7.5), 5 mM CaCl₂, 1 mM ZnCl₂]. The gel was stained with Coomassie brilliant blue R-250 to detect the gelatin-degraded unstained clear bands, which were in turn semiquantified by densitometric scanning using an Imagae Analyzer LAS-1000 plus (GE Healthcare Japan, Tokyo, Japan).

**RESULTS**

**Acteoside Promotes the Activation of proMMP-2 and the Expression of MT1-MMP in NHDF** Among the MMP isotypes, the expression and activation of proMMP-2 has been reported to be associated with cutaneous wound healing, because active MMP-2 participates in basement membrane remodeling, resulting in accelerating cell migration and proliferation during wound healing. We first examined the effects of acteoside on the production and activation of proMMP-2 in the presence or absence of IL-1β and TNF-α in NHDF. The mRNA expression of MMP-2 was slightly increased by the IL-1β-treatment (Fig. 2A). In addition, there was no change in MMP-2 gene expression as well as the production of proMMP-2 in the acteoside-treated NHDF (Figs. 2A, B). However, the activation of proMMP-2 was dose-dependently augmented by acteoside (Fig. 2B). In addition, the acteoside-induced proMMP-2 activation was detectable in a time-dependent manner (Fig. 3A). Furthermore, the acteoside-augmented activation of proMMP-2 was detectable in the
TNF-α-treated NHDF (Fig. S2).

Since proMMP-2 has been reported to be activated by MT1-MMP on the cell surface, we examined the effect of acteoside on the expression of MT1-MMP in NHDF. As shown in Fig. 3B, acteoside was found to time-dependently augment the mRNA level of MT1-MMP in NHDF. In addition, the IL-1β-augmented MT1-MMP gene expression was enhanced by acteoside at 24 h after treatment. Under these experimental conditions, we have confirmed that there was no acteoside cytotoxicity (6.25–100 µM) in NHDF (data not shown). Since it has been reported that concanavalin-A, which is a lectin from Canavalia ensiformis, increases proMMP-2 activation due to the augmentation of MT1-MMP expression in dermal fibroblasts from humans and rabbits, we further confirmed whether concanavalin-A increased proMMP-2 activation in NHDF. As shown in Fig. 2C, like in the case of acteoside, concanavalin-A facilitated proMMP-2 activation in NHDF. Therefore, these results suggest that acteosides augment the activation of proMMP-2 in concomitance with an increase in MT1-MMP expression in NHDF under both inflammatory and non-inflammatory conditions.

Catechols in Acteoside Are Crucial for the Augmentation of proMMP-2 Activation in NHDF

As acteoside is a glycoside with two aglycons such as CA and DOPE (I and II, respectively, in Figs. 1 and S1), we next examined whether such aglycons are associated with the acteoside-mediated proMMP-2 activation in NHDF. As shown in Fig. 4A, CA was found to enhance the IL-1β-augmented proMMP-2 activation in NHDF in a dose-dependent manner. In addition, the similar enhancement of proMMP-2 activation was observed by administrating DOPE. Both CA- and DOPE-enhanced proMMP-2 activation was detectable in the TNFα-treated NHDF (Fig. S2). However, neither MOPE nor HVA, in both of which a 3-hydroxy group in catechol was substituted for a methoxy group (Fig. S1), influenced the IL-1β-augmented proMMP-2 activation. Therefore, these results suggest that catechols in acteoside are associated with the acteoside-induced proMMP-2 activation in NHDF.

Involvement of the Phosphatidylinositol-3-Kinase Pathway in the Augmentation of proMMP-2 Activation by Acteoside

To determine the intracellular pathway(s) of the acteoside action leading to proMMP-2 activation, we examined the effect of inhibitors against several intracellular signal molecules on the acteoside-induced proMMP-2 activation in NHDF. As shown in Figs. 5A and B, there was no change in the acteoside-induced proMMP-2 activation in the IL-1β-stimulated NHDF in the presence of inhibitors: U0126, PD98059, SB203580, and SP600125 against mitogen-activating kinase kinase-1/2 (MEK-1/2), extracellular signal-regulated kinase (ERK)-1/2, p38 stress-activated protein kinase (SAPK), and c-Jun N-terminal kinase (JNK), respectively. However, a phosphatidylinositol-3-kinase (PI3K) inhibitor, LY294002, was found to inhibit the acteoside-mediated proMMP-2 activation (Fig. 5B). In addition, the CA- and DOPE-enhanced proMMP-2 activation was inhibited by LY294002 in the IL-1β-treated NHDF (Fig. S3). Furthermore, the IL-1β and acteoside-augmented MT1-MMP mRNA expression was diminished by administrating LY294002 to NHDF (Fig. 5C).
Fig. 3. Acteoside Accelerates proMMP-2 Activation Accompanied with MT1-MMP Gene Expression in NHDF

NHDF at the 18th passage were treated for 4–24 h with acteoside (100 μM) in the presence or absence of IL-1β (10 ng/mL). A: The harvested culture medium was subjected to gelatin zymography as described in Fig. 2. More than three independent experiments using the cells at different passages were highly reproducible and typical data are shown. Data are represented as means ± S.E.M. for three independent wells. iMMP-2, intermediate MMP-2. Active MMP-2, active form of MMP-2. B: Real-time RT-PCR for MT1-MMP gene expression was performed as described in Fig. 2. More than three independent experiments using the cells at different passages were highly reproducible, and typical data are shown. Data are represented as means ± S.E.M. for three independent wells. *, **, and *** Significantly different from untreated cells (control) at each time (p < 0.05, 0.01, and 0.001, respectively). # and ### Significantly different from the cells treated with IL-1β alone at each time (p < 0.05 and 0.001, respectively).

Fig. 4. Effects of Catechol Derivatives on the proMMP-2 Activation in NHDF

NHDF at the 18th passage were treated for 24 h with acteoside (ACT) (6.3–100 μM), caffeic acid (CA) (6.3–100 μM), 3,4-dihydroxyphenylethanol (DOPE) (6.3–100 μM), 4-hydroxy-3-methoxyphenylethanol (MOPE), and homovanillic acid (HVA) (6.3–100 μM) in the presence of IL-1β (10 ng/mL). The harvested culture medium was subjected to gelatin zymography as described in Fig. 2. More than three independent experiments using the cells at different passages were highly reproducible and typical data are shown. Data are represented as means ± S.E.M. for three independent wells. * and ** Significantly different from untreated cells (control) (p < 0.05 and 0.01, respectively). ## and ### Significantly different from the cells treated with IL-1β alone (p < 0.01 and 0.001, respectively). iMMP-2, intermediate MMP-2. Active MMP-2, active form of MMP-2.
Therefore, these results indicate that the PI3K pathway was preferentially and selectively associated with the acteoside-induced proMMP-2 activation and MT1-MMP gene expression in NHDF.

DISCUSSION

Coordinated ECM proteolysis has been involved in the process of wound healing, which is requisite for keratinocyte migration, fibroblast recruitment, and angiogenesis in the healing of wounds.\textsuperscript{1,3} Medicinal herbs have been used worldwide to heal cutaneous wounds. For instance, the Angelica species such as Angelica sinensis and Angelica dahurica have been reported to facilitate wound healing.\textsuperscript{20,21} In the present study, we demonstrated for the first time that acteoside isolated from the dry leaves of R. glutinosa L. is an attractive candidate to modulate wound healing by regulating proMMP-2 activation in NHDF. Furthermore, Akdemir et al.\textsuperscript{15} have reported that a phenylethanoid glycoside, verbascoside, from Verbascum mucronatum L. exhibits wound healing activity in vivo. Verbascoside has two aglycons: DOPE and CA, and is structurally similar to acteoside but saccharide. Thus, the previous report is likely to support our finding that acteoside is a potential candidate with wound healing activity.

Cutaneous wound healing is a rapid and effective process for the reconstitution of structural and functional barriers. In general, the wound healing process is divided into three phases: (1) inflammation, (2) re-epithelialization and granulation tissue formation, and (3) synthesis of ECM components and the degradation of discarded ones (remodeling). These processes are critically regulated by the functions of the cells including dermal fibroblasts, keratinocytes, and lymphocytes.\textsuperscript{1} Regarding inflammation, inflammatory cytokines such as IL-1\(\beta\) and TNF-\(\alpha\) have been reported to be involved in the re-epithelialization and granulation tissue formation in the wound healing process.\textsuperscript{1,17} Taken together with the knowledge that MT1-MMP-mediated proMMP-2 activation participates in the migration of keratinocytes and fibroblasts into the wound site,\textsuperscript{2,5} our findings that IL-1\(\beta\) and TNF-\(\alpha\) augment proMMP-2 activation may support that both IL-1\(\beta\) and TNF-\(\alpha\) at least play a crucial mediator role in wound repair in the skin.

It has been reported that MMP-2 degrades type IV collagen and denatured collagens (gelatin), resulting in the association with cellular migration and proliferation.\textsuperscript{22} Therefore, the activation of proMMP-2 is considered to be an essential process for early tissue repair during healing wound.\textsuperscript{2} However, the excess production of MMPs is considered to interfere with physiological tissue repair, leading to undesirable wounds with cosmetic and functional problems such as a fibrotic, contracted scars.\textsuperscript{23} In this study, we have demonstrated that acteoside suppressed the IL-1\(\beta\)-mediated MMPs-1 and -3 expression in NHDF (Fig. S4), indicating that the regulation of MMP expression by acteoside differs among MMP species. Furthermore, previous studies have reported that the excess levels of MMPs-1 and -3 result in chronic non-healing wounds.\textsuperscript{3,4} Thus, our results suggest that acteoside may prevent aberrant cutaneous tissue remodeling due to the inhibition of excess tissue breakdown by MMPs.
Regarding the mechanism(s) of acteoside-mediated biological activity, it has been reported that acteoside suppresses Ca²⁺-dependent CaMK (calmodulin-dependent protein kinase)/ERK and JNK/nuclear factor-kappaB (NF-κB)-signaling pathways. In addition, acteoside has been shown to reduce the production of intercellular adhesion molecule-1 through the inhibition of ERK and JNK in human vascular endothelial cells. Furthermore, it has been reported that the inhibition of activating protein-1 activation by acteoside results in a decrease in the production of nitric oxide and TNFα in macrophages. A recent study by Hwang et al. has shown that acteoside inhibits the phorbol ester-induced production of proMMPs-2 and -9, and MT1-MMP in human fibrosarcoma HT-1080 cells, resulting in a consequent decrease in cell invasion and migration in vitro. In the present study, we have demonstrated that a potent inhibitor against PI3K, but not MEK1/2, ERK, SAPK, and JNK, selectively inhibited acteoside-dependent proMMP-2 activation and MT1-MMP expression in NHDF. Therefore, the regulation of intracellular signaling pathway(s) by acteoside seems to be dependent on cell species. Nonetheless, a PI3K pathway may be associated with the acceleration of proMMP-2 activation and MT1-MMP expression by acteoside in NHDF.

Kurisu et al. have reported that acteoside, as well as CA, increases the production of hepatocyte growth factor (HGF) in NHDF, whereas such action is negligible in DOPE-treated cells. In the present study, we demonstrated that, like in the case of acteoside, both CA and DOPE facilitated proMMP-2 activation in the IL-1β and TNFα-treated NHDF. The proMMP-2 activation ratio by CA or DOPE is similar to that by acteoside. In addition, the CA- and DOPE-augmented proMMP-2 activation was suppressed by LY294002 in the NHDF. Furthermore, there was no change in the proMMP-2 activation by MOPE and HVA in the IL-1β-stimulated NHDF. Thus, catechols such as CA and DOPE, but not two sugars, in acteoside play an important role in the acteoside’s biological activity through a possible PI3K pathway. Moreover, both aglycons in acteoside may regulate independently HGF production and proMMP-2 activation in NHDF. In this regard, adrenergic receptor-dependent signals have been reported to accelerate cutaneous wound healing in vivo and in vitro. Therefore, acteoside may exhibit wound healing actions through an adrenergic receptor in NHDF. Further experiments are needed to confirm this hypothesis.

In conclusion, we have provided novel evidence that catechols, such as CA and DOPE, but not two sugars in acteoside, augment proMMP-2 activation along with an increase in MT1-MMP expression through a PI3K signal pathway in NHDF. Therefore, not only CA and DOPE, but also acteoside, are likely to be attractive candidates that facilitate cell migration and proliferation as well as granulation tissue formation in the skin wound repair process when the additional roles of two aglycons are elucidated.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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