**Plantago asiatica** Extracts Inhibit UV-induced Matrix Metalloproteinase-1 in Human Dermal Fibroblasts and Prevent Skin Photoaging in Hairless Mice

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In this study, we examined the antiwrinkle effects of *Plantago asiatica* seed (PAS) on UV-induced human dermal fibroblasts (HDFs) and hairless mice models to determine if an anti-inflammatory agent could also be developed as an antiaging treatment. Acute UV irradiation induced matrix metalloproteinase (MMP)-1 protein expression levels, but this was suppressed by PAS in HDFs. Next, we investigated the effect of PAS on UV-induced skin changes in hairless mice. Chronic UV exposure of the dorsal skin increased skin thickness and induced wrinkle formation. PAS significantly suppressed UV-induced morphologic skin changes. In addition, MMP-1 expression was dramatically attenuated by treatment with plantainoside D which was purely isolated from PAS, indicating that this is the principle compound inhibiting MMP-1 expression in HDFs. Taken together, our data suggest that PAS can prevent the harmful effects of UV that lead to skin aging.

**Keywords:** *Plantago asiatica*, Skin photo-aging, Matrix metalloproteinase, Plantainoside D

**Introduction**

Skin aging is a complex and progressive process that leads to functional and esthetic changes in the skin. This process can result from both extrinsic and intrinsic processes, and is commonly associated with increased wrinkling, sagging, and laxity. Intrinsic aging is due to chronologic damage caused by slow and irreversible tissue degeneration. Extrinsic aging is referred to as photoaging and is attributed to continuous, long-term exposure to ultraviolet (UV) radiation of approximately 300–400 nm.

UV irradiation usually damages the human skin and induces the expression of matrix metalloproteinases (MMPs) in fibroblasts. The upregulation of MMPs enhances the degradation of dermal collagen during UV-induced skin aging. MMPs are a family of zinc-dependent endopeptidases that play an important role in the remodeling of the extracellular matrix during developmental morphogenesis, angiogenesis, and tissue repair. They also play a role in tissue destruction during pathological processes, such as arthritic inflammation, skin aging, tumor invasion, and metastasis. MMPs are expressed at very low levels in unstimulated skin cells and healthy tissues, but some MMPs are induced by extracellular stimuli, such as UV, growth factors, cytokines, and tumor promoters. MMPs expressed in the human skin are regulated by inflammation-related transduction pathways, such as activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB). AP-1 forms heterodimer complexes with c-Jun and c-Fos induced by various inputs, including growth factors, cytokines, and UV exposure. NF-κB is an important MMP mediator that stimulates the transcription of proinflammatory cytokines such as IL-1β, TNFα, IL-6 and IL-8.

*Plantago asiatica* is a perennial plant that belongs to the Plantaginaceae family and is commonly used as a folk medicine in Korea, China, and Japan. The seed of *P. asiatica* (PAS) is used as an antihypertensive, antipyretic, antitussive, diuretic, expectorant, anti-inflammatory, and for would healing. In addition, *P. asiatica* possesses a broad-spectrum of antiviral, anticancer, and immunomodulation activities. PAS contains fatty acid, polysaccharides, aucubin, geniposidic acid, and phenylpropanoid glycosides as its main components. It has been reported that the main components of the herb, such as the phenylethanol glycoside plantamajoside, the flavonoid glycosides plantagin, and the irridoid glycoside aucubin, possess antibacterial, antiallergic, anti-inflammatory, antioxidation, and lipoxygenase inhibitory activities.

In this study, we investigated whether the topical application of PAS extract prevents UV-induced skin damage and attenuates the features of photoaging in mice. It was found that the topical application of PAS extract inhibited UV-induced decrease of collagen in hairless mice and attenuated UV-induced MMP-1 expression in dermal fibroblast. In addition, we explored the ability of the main component from *P. asiatica* to inhibits UV- or tumor necrosis factor-alpha (TNFα)-induced increase of MMP-1 and identified plantainoside D as an active component.

**Methods**

**Plant Material, Extraction, and Isolation.** The PAS (Plantaginaceae) were purchased from a local market (Kyungdong Herb-Market, Seoul, Korea) and authenticated by one of the authors (K. H. Kim). A voucher specimen
the UV source. The strength of the UVB light was measured with an energy peak at 312 nm was used as a Sankyo Denki G15T8E (Sankyo Denki, Kanagawa, Japan) indicated reagent concentrations 1 h before UV treatment. Cultured in serum-free medium for 24 h and pretreated with the n-butanol fraction (100 g) was subjected to Sepax GP-C18 ODS column chromatography using a water–methanol solvent system, and 20% methanol (44 g), 50% methanol (26 g) and 100% methanol (11 g) subfractions were obtained. Further separation with a 50% methanol fraction was completed with preparative HPLC chromatography (methanol-0.1% formic solution system) and gel column chromatography with methanol as an elute solvent to obtain the plantainoside D (55 mg). The physical and chemical data including UV, IR, 1H NMR, 13C NMR, HSQC, HMBC, and MS of plantainoside D were identical with those previously reported.

Cell Culture and UV Irradiation. Normal human dermal fibroblast (HDFs) cells were obtained from Lonza (Walkersville, MD, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories Inc., Logan, UT, USA) and 1% penicillin/streptomycin (Invitrogen) at 37 °C and 5% CO₂. HDFs were blocked in 5% skim milk at room temperature for 1 h and scratched with a 20 μl pipette tip to create a wound on the confluent monolayer. The total energy dose of UV irradiation was set to 20 mJ/cm² to optimize cell viability and MMP-1 induction.

Supernatants from cells were heated for 10 min at 95 °C and subjected to electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel. The proteins were transferred to Immobilon-P (Millipore, Billerica, MA, USA) and blocked in 5% skim milk at room temperature for 1 h and probed with the primary antibodies against MMP-1 (Calbiochem, Cambridge, MA, USA) for 24 h at 4 °C. After washing, bound antibodies were detected with corresponding horse radish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Signals were detected with Supersignal® West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) and were visualized with the LAS-4000 Luminescent Image Analyzer (Fuji Film, Tokyo, Japan). MMP-1 secretion was quantified from supernatants using a human MMP-1 ELISA Kit (Merck & Co. Inc., Whitehouse station, NJ, USA). The relative MMP-1 amount was normalized to the corresponding viability of cells determined by MTT assay (Ez-cytox, Dail Lab Service. Co., Seoul, Korea).

Animal Treatment. This study was conducted in conformity with the policies and procedures of the Institutional Animal Care and Use Committee of Sooam Biotech Research Foundation Co., Ltd. (IACUC 2011-8) and of the Institutional Animal Care and Use Committee of Soomkung Women's University. A female albino (6 week old) mouse was taken just before the animals were killed. Image analysis of the replicas was performed by automated (Image-Pro Plus, Media Cybernetics, Rockville, MD, USA) and visualized with the LAS-4000 Luminescent Image Analyzer (Fuji Film, Tokyo, Japan). Skin roughness, referred to as R1, is defined as the difference between the highest crest and lowest furrow. To exclude the possibility of artifact, the program cut the line into five equal parts: R3 represents an average of the maximum distance (R1) derived from each of the five parts of the line; R2 represents the largest value of these five distances; R4 represents the mean area surrounded by a horizontal line drawn at the highest crest and furrows profiles; and R5 stands for the mean deviation of the furrow's profile to the middle line. Skin specimens were obtained from dorsal of mice killed at the end of the study.

Tissue Preparation. Mouse dorsal skin samples were fixed in 10% buffered formalin, embedded in paraffin, and sectioned...
(3–4 μm) for light microscopy. Sections were stained with hematoxylin and eosin (H&E) and Masson-Trichrome (MT) for collagen fiber evaluation.

Statistical Analysis. The data are expressed as the means ± SD. Differences between the mean values in the two groups were analyzed using one-way analysis of variance (ANOVA). A p-value of <0.05 was considered statistically significant.

Results

PAS Extract Inhibited UV-induced Expression of MMP-1 in HDFs. Previous reports have shown that PAS significantly inhibited TNFα production and the translocation of NF-κB from the cytosol to the nucleus.22 Regulation of MMP-1 is primarily governed by transcription factor NF-κB that stimulates the transcription of proinflammatory cytokines, such as TNFα. To examine the effect of PAS on UV-induced expression of MMP-1, we measured the amount of MMP-1 HDFs secreted by western blot analysis and ELISA. Irradiation of UV led to about an eightfold increase in MMP-1 expression. However, pretreatment with the PAS extract attenuated UV-induced MMP-1 expression (Figure 1(a) and (b)). These results indicate that components in the extracts might prevent collagen degradation by MMP-1 in HDFs by inhibiting its expression.

PAS Extract Prevents UV-induced Wrinkle Formation in Hairless Mice. Next, we explored whether PAS can prevent UV-induced changes, including wrinkle formation in hairless mice. Hairless mice in the control group were treated with vehicle and no significant changes in wrinkle formation were observed over the 12 weeks of treatment. However, the repetitive exposure of vehicle-treated mice to UV radiation increased wrinkle formation compared to the vehicle-treated group not exposed to UV radiation at 12 weeks. Topical treatment with PAS extract significantly reduced wrinkle formation (Figure 2(a)). The results at 12 weeks were further confirmed by image analysis using skin replicas. Visiometer R-values R1 through R5 decreased as wrinkles diminished. As shown in Figure 2(b), there were significant differences in the skin roughness (R1), maximum roughness (R2), average roughness (R3), smoothness depth (R4), and arithmetic average roughness (R5) between the vehicle-treated group exposed to UV radiation compared to the vehicle-treated group exposed to UV radiation. Taken together, our results suggest that PAS can suppress UV-induced wrinkle formation by downregulating MMP-1.

PAS Extract Inhibited UV-induced Epidermal Thickening and Dermal Collagen Decrease in Hairless Mice. Since UV irradiation has been shown to induce epidermal thickening in skin, we investigated the effects of PAS on UV-induced epidermal thickening. PAS was applied topically to mouse dorsal skin and the skin was treated with increasing dose of UV (1 to 4MED) for 12 weeks. After irradiation, skin samples were obtained from the dorsal skin of killed mice and serially sectioned samples were stained with H&E. UV-induced epidermal thickening by twofold (p < 0.05 vs. vehicle group, n = 7), and topical PSA extract decreased it by 0.75-fold (p < 0.05 vs. UV group, n = 7) (Figure 3(b)). We investigated the effect of PAS on decreased collagen expression in UV-irradiated skin by using MT stain. Collagen fiber staining showed compact and dense structure in PAS-treated, UV-irradiated skin compared to vehicle-treated, UV-irradiated skin as revealed by MT stain (Figure 3(a)). These results demonstrate that the topical application of PAS inhibited the UV-induced decrease of collagen expression in vivo.

Plantainoside D Inhibits MMP-1 Expression in HDFs. Next, we tried to isolate the active compounds from P. asiatica to inhibit MMP-1 in HDFs. One compound was purified with solvent extraction, open column chromatography, and a preparative HPLC chromatography. Its structure was analyzed by $^1$H/$^{13}$C-NMR and mass spectrometry, and was identified as plantainoside D. Thus, we examined the effect of plantainoside D on MMP-1 expression in HDFs. Treatment with plantainoside D caused an attenuation of UV- or TNFα-induced expression of MMP-1 (Figure 4(a) and (b)). Other major compounds, such as acteoside and aucubin, did not show any
effects on UV or TNFα-induced MMP-1 expression (data not shown). These results strongly indicate that plantainoside D from *P. asiatica* is the major compound responsible for the reduction of MMP-1 expression in HDFs.

**Discussion**

Skin aging is the hallmark of prolonged UV exposure and intrinsic aging that causes collagen breakdown by increasing the expression levels of MMP enzymes. It has been repeatedly demonstrated that reducing MMP-1 is a potential strategy for treating and preventing the clinical manifestations of skin aging. The UV-induced signaling pathways in the epidermis and dermis appear similar to the inflammatory response. The major transcription factors in stimulated keratinocytes, fibroblasts, and melanocytes, such as NF-κB and AP-1, are also key proteins in inflammation. Thus, compounds that have anti-inflammatory activity are worth...
evaluating for their potential antiaging effects. Additionally, these compounds should be obtained and/or isolated for further research and biomedical application.

PAS has long been used to treat hypertension, ischialgia, asthma, and arthritis because of its anti-inflammatory activity. Additionally, this herbal material has been reported to contain various iridoids, such as aucubin, catalpol, and geniposide that inhibit COX-2, TNF-\(\alpha\), and nitric oxide by preventing the nuclear translocation of NF-\(\kappa\)B.22 Plantainoside D, contained in \(P. asiatica\), \(Chirita longgangensis\) var. \(hongyao\), \(Picrorhiza scrophulariiflora\), \(Digitalis purpurea\), and \(Penstemon linarioides\) showed antihypertensive, antioxidant, anti-inflammatory, and chemopreventive activities.17,26–29

In this study, we demonstrate the beneficial effect of PAS and one of its compounds, plantainoside D, in HDFs which could be developed as a topical agent used to prevent and treat skin aging. Treatment of HDFs with PAS extract decreased UV-induced expression of MMP-1 (Figure 1(a) and (b)), reduced wrinkle formation (Figure 2(a)), and restored collagen content in vivo (Figure 3(a)). In addition, our data suggest that plantainoside D is an active principle of \(P. asiatica\) that inhibits MMP-1 expression induced by UV or TNF-\(\alpha\) (Figure 4(a) and (b)). Plantainoside D derived from \(P. asiatica\) demonstrated
antioxidant and anti-inflammatory activities. In cell-based experiments to determine antiwrinkle activity, PAS extract inhibited UV-induced MMP-1 expression in HDFs. TNFα and growth factors are overexpressed following internal and external stress. They can activate dermal fibroblasts and over-express the MMP-1 protein, which breaks down collagen in the extracellular matrix.30 Specifically, PAS extract inhibited activation and nuclear translocation of NF-κB.31 These results suggest that the inhibition of NF-κB activation reduces transcription of TNFα and expression of MMP-1. NF-κB is the main transcription factor in the UVB-stimulated signaling pathway and PAS extract can reduce NF-κB activation and the release of TNFα and MMP-1 in fibroblasts.

Dermal fibroblasts are either directly activated by short-wavelength UV or indirectly activated through TNFα released from keratinocytes.31 To determine the paracrine system in the release of TNFα main transcription factor in the UVB-stimulated signaling

References