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BRIEF REPORT

The Protective Effect of *Ganoderma lucidum* Extract in Ultraviolet B-Induced Human Dermal Fibroblasts and Skin Equivalent Models

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Dear Editor:

Ultraviolet (UV) radiation is a main cause of skin damage, including photoaging, generation of reactive oxygen species (ROS) and DNA damage. Photoaging in skin involves structural and functional modifications of the extracellular matrix (ECM) caused by the upregulation of the expression of matrix metalloproteinases (MMPs) and the downregulation of collagen[1,2]. Therefore, many studies have focused on controlling the amount of MMPs and collagen in UV-induced cells for treatment.

Ganoderma lucidum has been used in China as a herbal medicine. Several studies have suggested that *G. lucidum* polysaccharides have anti-oxidative effect by activating the antioxidant enzyme activities and reducing ROS induced by ultraviolet B (UVB) irradiation[3]. However, the UVB irradiation protective effect of *G. lucidum* is less understood. In the current study, therefore, we investigated the protective effect of *G. lucidum* extract (GLE) in UVB-irradiated neonatal human dermal fibroblasts (HDF) and skin-equivalent models. Specifically, HDF cells irradiated with UVB were treated with GLE, and the levels of MMP-1 and procollagen were compared. Using a nonlinear optical imaging microscopy technique including second-harmonic generation (SHG) and two-photon excitation fluorescence (TPEF), we visualized morphological changes in UVB-irradiated HDF cells and their surrounding collagen fibrils in skin equivalent (SE) models.

Firstly, we examined whether GLE (Fig. 1A) had a cytotoxic effect on HDF. Treatment with GLE (12.5～200 μg/ml) into HDF for 48 hours did not induce cytotoxicity (Fig. 1B). We investigated whether GLE inhibited MMP-1 secretion in UVB-irradiated HDF cells. HDF cells were irradiated with 30 mJ/cm^2 of UVB and then treated with GLE for 48 hours. GLE inhibited MMP-1 secretion in UVB-irradiated HDF cells (Fig. 1C). To investigate whether GLE induces procollagen synthesis in the presence of UVB irradiation (30 mJ/cm^2), we performed a type I procollagen enzyme-linked immunosorbent assay (ELISA) after treatment with GLE. The results showed that GLE significantly increased type I procollagen synthesis in a dose-dependent manner (Fig. 1D). To further discover the molecular mechanism of GLE, we focused on mitogen-activated protein kinases (MAPK) activation because the MAPK (ERK/JNK/p38) signaling pathway is well known for regulating transcriptional activation of MMP-1 in UVB-irradiated HDF[4]. Therefore, we used Western blot analysis to identify the MAPK

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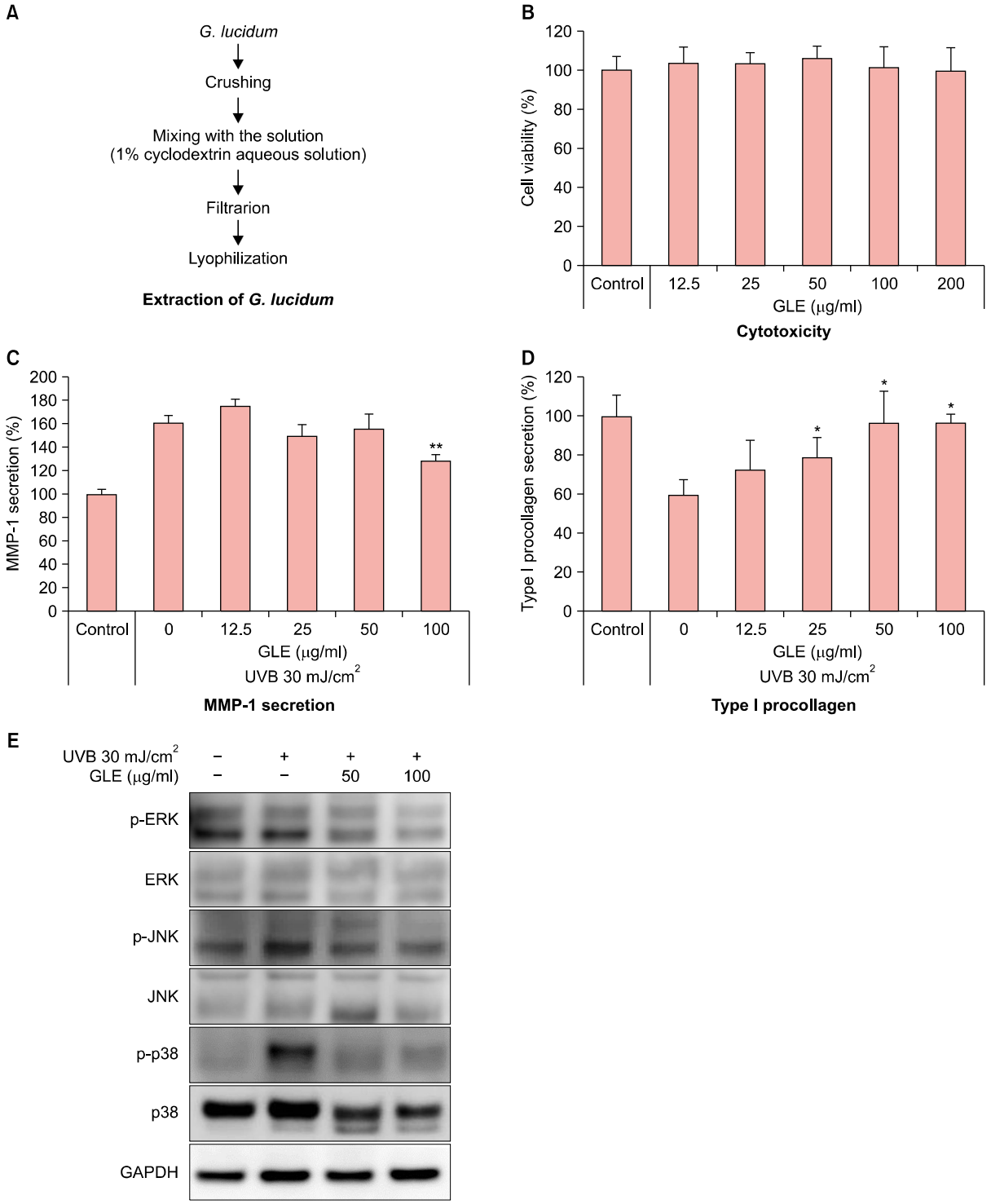

Fig. 1. (A) Extraction procedure of *Ganoderma lucidum* extract (GLE). (B) Neonatal human dermal fibroblasts (HDF) cells were treated with different concentrations of GLE for 48 hours and then cell viability was quantified by CCK8 assay. (C) HDF cells were irradiated by ultraviolet (UV) B (30 mJ/cm^2) and treated with different concentrations of GLE for 48 hours. The level of matrix metalloproteinases (MMP)-1 secretion into the culture media was quantified by MMP-1 enzyme-linked immunosorbent assay (ELISA). Results were relatively quantified after normalizing the number of viable cells based on a cell-viability assay. (D) Type I procollagen secretion of HDF cells after treatment with GLE was quantified by ELISA in 30 mJ/cm^2 of UVB-irradiated condition. Results were relatively quantified after normalizing the number of viable cells based on a cell-viability assay. (E) HDF cells were pretreated with different concentrations of GLE for 3 hours, and then cells were irradiated by UVB (30 mJ/cm^2). Cell lysates were harvested 1 hours after UVB irradiation. The level of proteins involved in UVB-downstream signaling pathways was analysed via Western blotting. *$p<0.05$, **$p<0.01$.

signaling pathway (Fig. 1E). UVB radiation induced phosphorylation of all three signaling molecules (ERK/JNK/p38), as is consistent with previous studies[5]. Interestingly, treatment with GLE attenuated phosphorylation of ERK, JNK, and p38 in a dose-dependent manner. Among them, p38 kinase was the most influenced by the treatment with GLE.

Next, we made a human SE containing keratinocytes in epidermis and fibroblasts in dermis, as we previously reported[6], and used SEs to examine whether GLE has a similar effect on HDF cells in a 3-dimensional (3D) environment. The SEs were exposed to UVB (40 mJ/cm^2) and treated with GLE (100 μg/ml) for 48 hours. Then the SEs were fixed and stained with 1 μM of CellTracker^{TM} Red (CMPTX; Invitrogen, Carlsbad, CA, USA) to visualize the dermal fibroblasts. Finally, collagen fibrils and the fluorescence-labeled cells in the SEs were visualized by a multiphoton microscopy, which is implemented in two different contrast mechanisms, TPEF and SHG. TPEF and SHG have shown several advantages over conventional confocal imaging technique, such as low phototoxicity, deep penetration depth, and being label-free. TPEF has been used for imaging endogenous autofluorescence (melanin, keratin, and elastin) and exogenous fluorescence dyes in biological samples[7]. Unlike fluorescence-based imaging, SHG detects specific molecules with noncentrosymmetric molecular assemblies, such as collagen, myosin, and microtubules[8,9]. Recently, a combination of TPEF and SHG has been evaluated for the studies of interactions between cells and the ECM in a 3D environment[10]. Therefore, using a cytoplasmic staining dye and a combined TPEF/SHG imaging technique, we simultaneously visualized intact collagen fibrils by SHG imaging in a label-free manner and fluorescence-stained dermal fibroblasts by TPEF imaging in a labeled manner. As shown in Fig. 2, there was a significant decrease in the density of collagen fibrils in the dermis of the UVB-irradiated SEs. When GLE was treated with the UVB-irradiated SEs for 48 hours, collagen fibrils were enriched in areas that were axially and laterally ad-

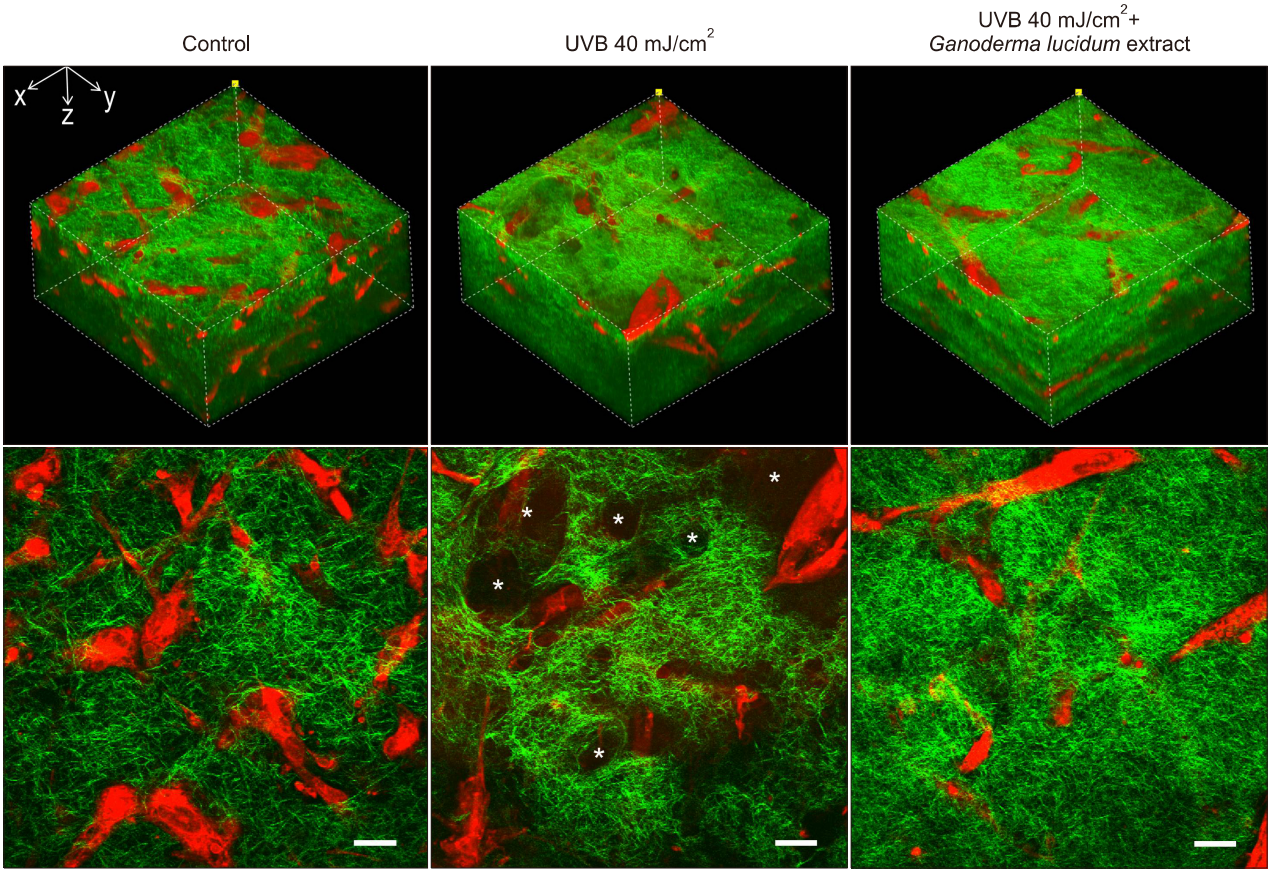

Fig. 2. A 3-dimensional (3D) two-photon excitation fluorescence (TPEF)/second-harmonic generation (SHG) imaging for the dermis of human skin equivalents. The total measurement volume (upper panel) is 200 (x)×200 (y)×100 (z) μm^3. Red indicats the TPEF signals for fluorescence-stained human dermal fibroblasts cells, and green indicates the SHG signals for collagen fibrils. The bottom panels indicate representative 2D images. Asterisks indicate the disrupted hole-like structures of collagen fibrils caused by ultraviolet B irradiation. Scale bars = 20 μm.

jacent to the HDF cells. Namely, we obviously distinguished the dermis with collagen fibrils densely packed in control sample, from the dermis with thicker fibrils in a more opened structure like the hole (indicated by an asterisk in Fig. 2). However, GLE retained the collagen fibrils in the UVB-irradiated SEs. The formation of hole-like structure is related with collagen degradation[10], which could be caused by UVB-induced photoaging mechanism (increased expression of MMP in fibroblast by UV exposure). Importantly, GLE attenuate the formation of hole-like structure and MMP-1 expression in our study, therefore, the results support that GLE has potential effectiveness in UVB-induced photoaging.

In this study, we investigated the protective effects of GLE on UVB-induced skin aging in HDF cells and SE models. UVB radiation is one cause of extrinsic skin aging and induces upregulation of MMPs and downregulation of collagen. MMPs, which are generated from HDF cells, can degrade components in the ECM, including collagen and elastin. Recently, Zeng et al.[3] reported that *G. lucidum* polysaccharides protect UVB-irradiated HDF cells. However, there is no evidence to show the underlying molecular mechanisms of *G. lucidum* polysaccharides in the study. In addition, they have no data using 3D SE.

Results from our study indicated that GLE inhibited UVB-induced MMP-1 expression and increased procollagen expression in HDF cells. We also determined for the first time that the activated MAPK signaling, especially p38, was inhibited by treatment with GLE in UVB-irradiated HDF cells. Furthermore, using the SE models which have been widely used to study skin physiology, we confirmed that the treatment with GLE retained the collagen structure in UVB-induced SEs analyzed by TPEF/SHG imaging.

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SUPPLEMENTARY MATERIALS

Supplementary data can be found via http://anndermatol.org/src/sm/ad-32-251-s001.pdf.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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