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병이소초 추출물의 항산화 및 MMP 발현 저해 효과

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Inhibitory Effect of *Ophioglossum vulgatum* on Free Radical and MMP Expression in UV-irradiated Human Dermal Fibroblasts

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요 약: 자외선에 의한 활성산소 종의 생성과 피부 광노화 촉진은 피부표면 조직의 파괴와 피부염증유발, 홍반 등을 수반하며, 일상생활에서 항상 생활 자외선에 노출되어 있는 피부는 이러한 위험 요인과 직면하고 있다. 병이소초추출물의 유해 라디칼 소거 효과 실험 결과 IC_{50} values가 18.2 μg/mL로 superoxide 소거 효과가 우수하게 나타났으며, 세포 내에서 ROS에 의해 형광을 띠는 물질로 전환되는 CM-DCFDA를 이용하여 ROS의 양을 측정한 결과 자외선에 의해 증가된 세포 내 ROS의 양이 병이소초 추출물을 처리함으로써 100 μg/mL 농도에서 30 % 이상의 우수한 소거효과를 나타내었다. 섬유아세포에 자외선(UVA) 6.3 J/cm^{2}으로 조사하고 병이소초 추출물을 처리한 결과, 자외선에 의해 높아졌던 MMP-1 단백질 발현량이 농도의존적으로 감소하였으며, 50 μg/mL의 농도에서 37.7 % 정도의 MMP-1 발현 억제효과를 나타냈다. RT-PCR 실험에서는 병이소초 추출물을 10 ~ 50 μg/mL 농도로 처리한 결과, 자외선에 의해 높아졌던 MMP-1 mRNA의 발현이 농도 의존적으로 확연히 감소함을 확인할 수 있었다. 따라서 병이소초추출물은 자외선에 의해서 발생할 수 있는 피부손상에 대하여 효과적으로 보호할 수 있는 우수한 항산화 및 피부세포 보호소재로 적용될 수 있다.

Abstract: Human skin is constantly exposed to environmental irritants such as smoke, chemicals and ultraviolet (UV). Free radicals and reactive oxygen species (ROS) caused by these environmental irritants play critical roles in cellular damage. In this study, to investigate the skin cell protective effect of *Ophioglossum vulgatum* extract, we investigated its effects on intercellular antioxidative activity and UVA-induced MMP expression in human dermal fibroblasts (HDFs). The dried *O. vulgatum* was extracted in a mixture of ethanol and water (1 : 1) for 24 h at room temperature. The extract was filtered and concentrated *in vacuo* and lyophilized. For testing intracellular ROS scavenging activity the cultured HDFs were analyzed by increase in DCF fluorescence upon exposure to UVB 20 mJ/cm^2. After treatment of *O. vulgatum* extracts, intracellular ROS levels were measured by luminescence spectrophotometer. Enzyme linked immuno sorbent assay (ELISA), and RT-PCR techniques were used for evaluating the effects of *O. vulgatum*on on MMP protein and mRNA expression in UVA irradiated HDFs. *O. vulgatum* extract was found to have ROS scavenging activity with the IC_{50} values of 18.2 μg/mL against superoxide radicals in the xanthine/xanthine oxidase system. After treatment of *O. vulgatum* extracts, the oxidation of CM-DCFDA

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was inhibited effectively and *O. vulgatum* extracts showed a potent free radical scavenging activity by 30.4 % at 100 μg/mL in UVB-irradiated HDFs. UVA induced MMP protein expression was reduced 37.7 % by treatment with *O. vulgatum* extract, and MMP-1 mRNA expression was reduced in a dose-dependent manner. Taken together, these results suggest that *O. vulgatum* extract prevents the skin cell damage induced by UV irradiation, and implies that *O. vulgatum* extract may be useful as a new ingredient for anti-aging cosmetics.

Keywords: *ophioglossum vulgatum, antioxidant, ultraviolet, matrix metalloproteinase, anti-aging*

1. Introduction

The skin aging process can be divided into photo-aging and intrinsic aging. The skin is the most susceptible organ to be damaged by ultraviolet (UV) irradiation as it is directly exposed to UV light. Damage to human skin due to repeated exposure to UVA (320 ~ 400 nm), UVB (280 ~ 320 nm) radiation and damage occurring as a result of the passage of time are considered to be distinct entities rather than similar skin aging processes[1]. Extracellular matrix (ECM) macromolecules are important for creating the cellular environments required during development and morphogenesis. Matrix metalloproteinases (MMPs) are necessary for tissue remodeling and the healing cascade normal physiological condition. The expression of MMPs in UV-irradiated fibroblasts is known to be initiated by reactive oxygen species (ROS) and by activation of a cell surface growth factor and cytokine receptors. With increasing age, collagen synthesis becomes lower and MMP-1 levels become higher in sun-protected human skin *in vivo*. UV irradiation induces the synthesis of MMP in human skin fibroblasts *in vitro* and MMP-mediated collagen destruction accounts, in large part, for the connective tissue damage that occurs in photoaging[2].

A number of antioxidants that possess oxygen radical scavenging properties have been tested as potentially beneficial photoprotective agents from these extrinsic factors[3]. Intrinsic oxidation-protecting enzyme systems, including superoxide dismutase (SOD), catalase, and glutathione peroxidase, and food-derived substances such as tocopherols, flavonoids, ascorbic acid and carotenes are known to diminish the undesired effects caused by oxidation processes in organisms[4].

Some antioxidants occurring naturally in plants have begun to receive much attention because people and animals regularly consume them. In particular, some plants show remarkable effect for a wide range of pharmacological use for anti-allergies, anti-inflammation and anti-oxidation[5].

In this study, to investigate the skin cell protective effect of *Ophioglossum vulgatum* extract, we investigated the effect of antioxidative activity and the inhibitory effect of MMP expression in UV-irradiated human dermal fibroblasts.

2. Materials and Methods

2.1. Materials and Preparation of Plant Extract

Nitroblue tetrazolium (NBT), butylated hydroxytoluene (BHT) and other chemicals were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). The fluorogenic probes, 5-(6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2 DCFDA) and pluronic F-127 were purchased from Molecular Probes (Eugene, OR, USA). *Ophioglossum vulgatum* was supplied from a standardized extraction company of Chinese traditional medicine, Shuang-Lin Herbal Extraction Co., Ltd., Ning-Bo, China. The plant material was identified by Prof. YH Zhang, Peking University. The plant was extracted in a mixture of ethanol and water (1 : 1) for 24 h at room temperature and the residue was further extracted for additional 24 h. The extract was filtered and concentrated *in vacuo* at 24 ℃ and lyophilized.

2.2. Measurement of Superoxide Radical Scavenging (NBT test)

Superoxide dismutase (SOD) activity was measured

by using xanthine-xanthine oxidase system as a source of superoxide and nitroblue tetrazolium as a scavenger for this radical. SOD activity was determined as described by K. Furuno *et al.*[6].

2.3. Cell Cultures and UV Irradiation

Human dermal fibroblasts (HDFs) from new born foreskin were acquired from Modern Tissue Technology (MTT, Korea). Human dermal fibroblasts were maintained in Dulbecco's Modified Eagle's Media (DMEM)/Ham's F12 with 10% fetal bovine serum (FBS) and kept in a humidified 5 % CO_2 atmosphere at 37 ℃ human dermal fibroblasts from passage 6 to 10 were used in the experiments. UV irradiation doses were 6.3 J/cm^2 (UVA) and 20 mJ/cm^2 (UVB), and the radiation intensity was measured by using UV radiometer (International light Inc., MA, USA).

2.4. Free Radical Scavenging Assay in Human Dermal Fibroblasts

HDFs (1.5×10^5/mL) were seeded into well plates and cultured overnight. CM-H_2DCFDA (molecular probe, Eugene, OR, USA) is able to react with free radical compound and generate fluorescent dichlorofluorescein (DCF). For the detection of ROS, HDFs were loaded with 4.0 μM CM-H_2DCFDA plus 2 % Pluronic F-127 in HEPES-buffered control salt solution (HCSS) containing 120 mM NaCl, 5 mM KCl, 1.6 mM $MgCl_2$, 2.3 mM $CaCl_2$, 15 mM glucose, 20 mM HEPES and 10 mM NaOH. The cells were incubated for 20 min at 37 ℃ and directly added test sample with various dosages. After 30 min at 37 ℃ the cells (HDFs) were irradiated by UVB source. Fluorescence was determined using a luminescence spectrophotometer (Perkin Elmer, UK) with an excitation wavelength of 488 nm and emission wavelength of 525 nm[7].

2.5. ROS Imaging using Confocal Microscopy

HDFs were treated with several dosages of test samples for 2 h, and were irradiated by UVB source (20 mJ/cm^2). HDFs grown on a glass-bottom dish were loaded with 1 μM CM-H_2DCFDA. The cells were incubated for 20 min at 37 ℃ and the fluorescence signal of DCF (Ex = 488 nm : Em = 525 nm), the oxidation product of CM-H_2DCFDA by free signals, was analyzed Leica DM IRE2 inverted microscope (Leica, Germany). To minimize background signal caused by direct oxidation of CM-H_2DCFDA by illumination at 488 nm, intracellular levels of ROS were analyzed within 3 sec after illumination using a 20 × HCX FLUOTA lens / TCS-SP2 confocal system (Leica, Germany).

2.6. Determination of MMP-1 by ELISA

Human dermal fibroblasts (3×10^4 cells/well) were seeded into 48-well plates and cultured overnight. The culture media were replaced with DMEM/Ham's F12 containing sample. After 24 h cultivation, MMP-1 secreted into the cultured medium was detected by enzyme-linked immunosorbent assay (ELISA) method.

2.7. Semiquantitative RT-PCR

The total RNA isolated by using RNeasy Mini Kit (Qiagen, MD, USA) according to the manufacturer's protocol. A volume of 1 μg total RNA from each sample was subjected to reverse transcription using Omniscript RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplification was performed in a reaction volume of 25 μL of cDNA product and HotStarTaq^{TM}DNA polymerase (Qiagen, Hilden, Germany) using automatic heat-block DNA thermal cycler (ASTEC PC801, ASTEC Inc, Tokyo, Japan). Amplification of the constitutively expressed GAPDH was used as an internal control to assess the reverse transcription efficiency. The oligonucleotide primers used were: 5′-AAA GGG AAT AAG TAC TGG GC-3′ (sense) and 5′-AAT TCC AGG AAA GTC ATG TG-3′ (antisense) for MMP-1 : 5′-ATG CAG AAG GAG ATC ACT GC-3′ (sense) and 5′-CTG CGC AAG TTA GGT TTT GT-3′ (antisence) for GAPDH. The temperature cycling condition of amplification were as follows: for MMP-1 and GAPDH, 15 min at 94 ℃ then 28 cycles of 94 ℃ for 30 s, 50 ℃ for 30 s, 72 ℃ for 60 s, and final extension at 72 ℃ for 10 min. PCR products were electrophoresed on a 1.5 % agarose gel and visualized by

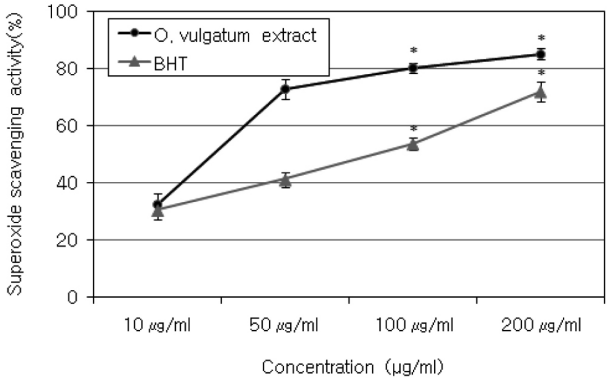

Figure 1. Superoxide scavenging activity. BHT was used as a positive control. The activity indicated by percentage of increase in comparison with that of control. The activity is significant (*$p < 0.05$) and the values are mean ± S.D. from 5 individual experiments.

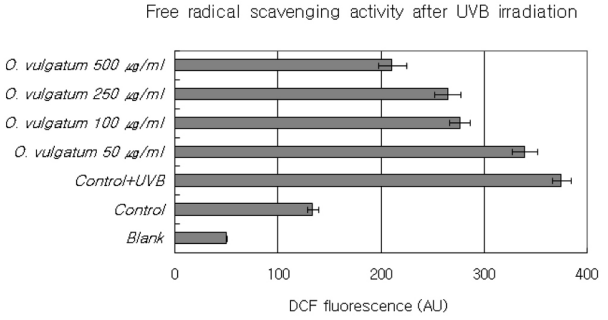

Figure 2. Effect of *O. vulgatum* on the production of intracellular ROS in HDFs. The cells were incubated with 4 μM CM-H_2DCFDA for 20 min, and irradiated by UVB 20 mJ/cm^2. ROS generation was assessed by luminescence spectrophotometer. The results are mean of triplicate samples with S.D.

ethidium bromide staining.

2.8. Statistical Analysis

The statistical significance of the results was analyzed by Student's *t*-test for unpaired observations.

3. Results and Discussion

3.1. Superoxide Scavenging Effect of *O. vulgatum* Extract

The superoxide radical (O^{-2}) is a highly toxic species which is generated by numerous biological and photochemical reactions. Results from the NBT analysis revealed that *O. vulgatum* extract exhibits scavenging potential for superoxide radicals with IC_{50} value of 18.2 μg/mL and showed more potent than BHT (IC_{50} 91.2 μg/mL)(Figure 1).

3.2. Free Radical Scavenging Activity After UV Irradiation in HDFs

For testing intracellular ROS scavenging activity, oxidative stress was also analyzed by following the increase in DCF fluorescence upon exposure to UVB 20 mJ/cm^2. In this study, the basal and UVB-induced levels of fluorescence for CM-H_2DCFDA loaded HDFs after the *O. vulgatum* extract treatment are shown in Figure 2. The basal level of ROS in HDFs in the normal culture condition was about 120 ~ 140 (AU), while that of CM-H_2DCFDA solution was about 50 (AU) in the same condition ($p < 0.05$). UVB exposure (20 mJ/cm^2) produced an increase about 2.8 fold over basal levels (fluorescence value : 375 AU) in fluorescence in CM-H_2DCFDA -loaded cells. After treatment with the extract in the culture medium for 2 h, the value of DCF fluorescence decreased remarkably in the dose dependent manner.

3.3. ROS Imaging using Confocal Microscopy

In this study, we observed green fluorescence intensity to determine free radical scavenging activity of *O. vulgatum* extract using CM-H_2DCFDA in cultured fibroblasts. In HDFs without UVB exposure, little fluorescence induction was noticed (basal level). Upon UVB-irradiated condition, we observed an induction of ROS to the bright green fluorescence in the cultured fibroblasts. By confocal microscope, we found out that newly induced ROS appeared in the nucleus and cytoplasm (see the green labeling in Figure 3). For irradiation of UVB 20 mJ/cm^2, intensity of green fluorescence is higher comparing to non-irradiated cells. After the treatment of the *O. vulgatum* extract at 50 μg/mL, we observed decrease of ROS induction compared to untreated cells after UVB irradiation. Thus, the low intensity of the green fluorescence was highly suggestive of a ROS scavenging effect for the extract.

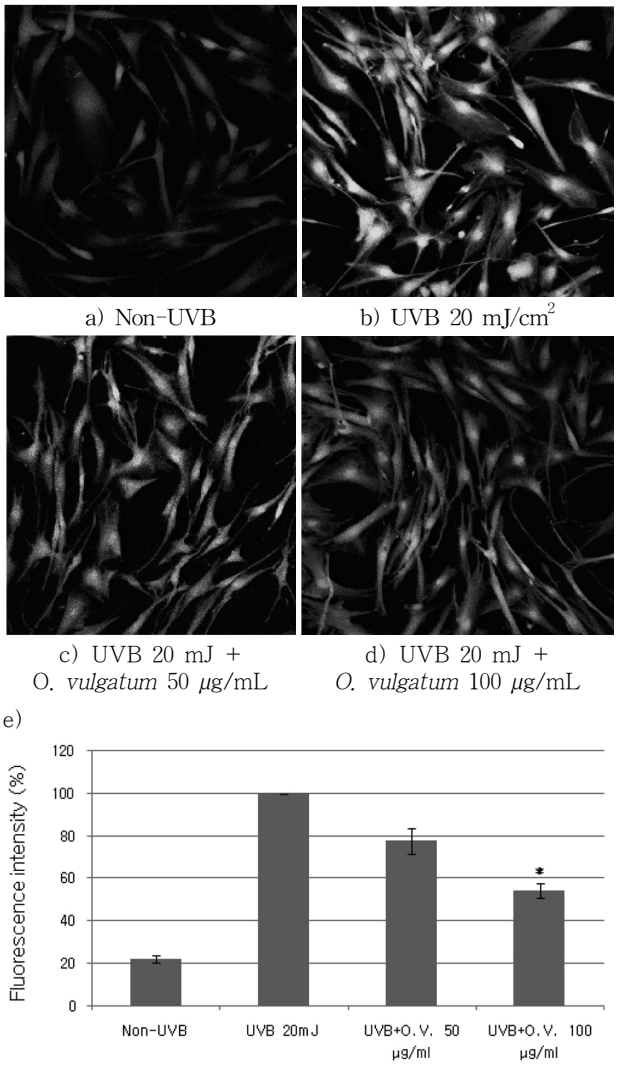

Figure 3. Confocal microscopic observation of ROS by CM-H_2DCFDA staining in cultured human dermal fibroblasts (HDFs). : (a) Control : HDFs on non-UVB, (b) exposed to 20 mJ/cm^2 UVB-irradiation, (c) pretreated with 50 μg/mL *O. vulgatum* for 2 h, (d) pretreated with 100 μg/mL *O. vulgatum* for 2 h, followed by treatment with 20 mJ/cm^2 UVB-irradiation, Magnification : × 400. (e) shows quantification of results from four independent experiments (a-d). The fluorescence intensity is significant (*$p < 0.05$) and the values are mean ± S.E. from 3 individual experiments.

3.3. Effect of *O. vulgatum* Extract on UVA-induced MMP-1 Expression

UV irradiation damages human skin and causes premature skin aging (photoaging) through the activation of MMPs which are responsible for the degradation of collagen, gelatin and other components of the ECM[8]. To examine the effect of *O. vulgatum* Extracton the expression of MMP-1 in human dermal fibroblasts, we exposed cultured fibroblasts to 6.3 J/cm^2 using UVA light, and measured the MMP-1 protein levels by ELISA. After treatment with *O. vulgatum* extract, MMP-1 protein expression was reduced 37.7 % at 50 μg/mL in UVA-irradiated HDFs (Figure 4).

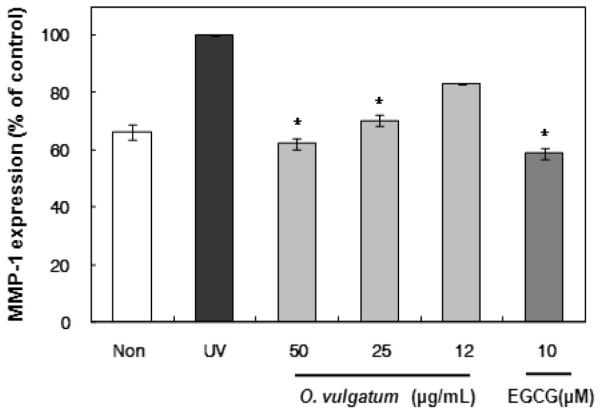

Figure 4. Inhibitory effect of the expression of MMP-1 in the UVA irradiated Human Dermal Fibroblasts (HDF). * $p < 0.05$ compared with UVA (UVA: 6.3 J/cm^2).

HDF cells were treated with various concentration of *O. vulgatum*extract for 24 h. Total RNA extracted from HDF was analyzed by semi-quantitative RT-PCR. After treatment with *O. vulgatum* extract, MMP-1 mRNA expression was reduced in a dose-dependent manner (Figure 5).

Theses results demonstrate that *O. vulgatum* extracts reduce the expression of MMP-1 at both the mRNA and protein levels. It is well established that the UV irradiation of cultured human skin *in vitro* or human skin *in vivo* induces the expression of MMPs which play important roles in the degradation of extracellular matrix components during premature skin aging[9]. Therefore, the development of MMP inhibitors has been considered to be promising strategy for therapy of UV stressed skin aging.

4. Conclusion

We found that *O. vulgatum* extracts had a potent

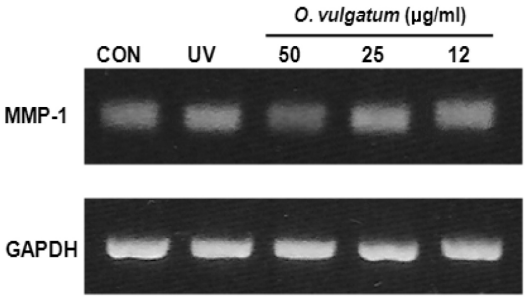

Figure 5. The effects of the extract on MMP-1 mRNA expression in the UVA irradiated Human Dermal Fibroblasts (HDF).

radical scavenging effect in cultured HDFs after UVB irradiation. In the study of MMP-1 expression, the extract had an inhibitory effect of MMP-1 mRNA and protein expression in cultured HDFs after UVA irradiation. These studies suggest that *O. vulgatum* extracts may also prevent the progression of photoaging of skin.

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