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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinical characteristics of patients with melanoma of the first cohort.

Table S2. LRAT and RPE65 staining intensities in melanocytic nevi and melanoma metastases.

Data S1. Material and methods.

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Letter to the Editor

Dihydroavenanthramide D prevents UV-irradiated generation of reactive oxygen species and expression of matrix metalloproteinase-1 and -3 in human dermal fibroblasts

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Abstract: Ultraviolet B (UVB) radiation induces photoageing by upregulating the expression of matrix metalloproteinases (MMPs) in human skin cells. Dihydroavenanthramide D (DHA_vD) is a synthetic analog to naturally occurring avenanthramide, which is the active component in oats. Although anti-inflammatory, anti-atherosclerotic and antioxidant effects have been reported, the anti-photoageing effects of DHA_vD are yet to be understood. In this study, we investigated the inhibitory effects of DHA_vD on UVB-induced production of reactive oxygen species (ROS) and expression of MMPs, and its molecular mechanism in UVB-irradiated human dermal fibroblasts. Western blot and real-time PCR analyses revealed that DHA_vD inhibited UVB-induced

MMP-1 and MMP-3 expression. It also significantly blocked UVB-induced ROS generation in fibroblasts. Additionally, DHA_vD attenuated UVB-induced phosphorylation of MAPKs, activation of NF- κ B and AP-1. DHA_vD regulates UVB-irradiated MMP expression by inhibiting ROS-mediated MAPK/NF- κ B and AP-1 activation. DHA_vD may be a useful candidate for preventing UV light-induced skin photoageing.

Key words: DHA_vD – MAPK – MMP – ROS – UVB

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Background

Ultraviolet (UV) radiation in sunlight is the major environmental cause of skin damage (1). Interactions between UV and molecular components of the epidermis and dermis cause acute and

chronic cutaneous changes called photoageing (2). Chronic exposure of human skin to UVB radiation induces the production of reactive oxygen species (ROS) and matrix metalloproteinases (MMPs) (3).

ROS are generated in response to UV irradiation and act as second messengers in signalling pathways and in the regulation of gene expression by activating transcription factors, such as activator protein-1 (AP-1) and nuclear factor- κ B (NF- κ B), leading to cellular oxidative stress (4). These transcription factors are regulated by mitogen-activated protein kinases (MAPKs) (5,6), which increase MMP expression.

Dihydroavenanthramide D (DHA_vD) is a synthetic analog to the naturally occurring avenanthramide, which is the active component in oats (7). Avenanthramide is effective for reducing atherosclerosis (8), inflammation (9) and oxidative stress (10). Recent studies have shown that DHA_vD inhibits NF- κ B activation (11,12). However, the anti-photoageing effects of DHA_vD have not yet been reported. In the present study, we evaluated the preventive effects of DHA_vD on UVB-induced production of ROS and expression of MMPs and investigated its molecular mechanism in UVB-irradiated HDFs.

Question addressed

We evaluated the anti-skin photoageing effects of DHA_vD by confirming its inhibitory effect on UVB-induced MMP expression through inhibition of ROS and MAPK/NF- κ B/AP-1 in HDFs.

Experimental design

Isolation and culture of HDFs

HDFs were isolated aseptically from human foreskin. Dermal fibroblasts that spread as a radial outgrowth from the attached pieces of dermis were cultured in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum and 1% antibiotics, at 37°C in a 5% CO₂ incubator.

UV irradiation

HDFs were rinsed twice with PBS and irradiated using a UVB cross-linker (Model CL-508M; Vilber Lourmat, Paris, France). Immediately after irradiation, fresh serum-free medium was added to the HDFs. Responses were measured after incubation for each experimental condition. The same schedule of medium changes without UVB irradiation was followed for the control cells.

Determination of MMP-1 and MMP-3 expression using Western blotting and an enzyme-linked immunosorbent assay

HDFs (2.0×10^6 cells) were irradiated with UVB (25 mJ/cm²), treated with DHA_vD (SymCalmin[®]) (Symrise GmbH & Co, Holzminden, Germany) for 24 h and lysed using 40 μ L ice-cold M-PER[®] Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA). The samples were separated using SDS-PAGE.

Active MMP-1 in the culture supernatants was quantified using a Fluorokine E Human Active MMP-1 Fluorescent Assay Kit (R&D Systems, Minneapolis, MN, USA); the MMP-3 level in the cell culture supernatants was determined using a Quantikine ELISA Kit (R&D Systems), according to the manufacturer's protocol.

Quantification of intracellular ROS

The intracellular concentration of ROS in HDFs was measured using an oxidation-sensitive fluorescent probe dye, DCF-DA. DCF fluorescence was detected using a FACStar flow cytometer (Becton Dickinson).

Electrophoretic mobility shift assay

Activation of NF- κ B and AP-1 was assayed with an EMSA using nuclear extracts.

Statistical analysis

Statistical analysis was performed using analysis of variance and Duncan's test. *P*-values < 0.05 were considered statistically significant.

Results and discussion

DHA_vD inhibits UVB-induced MMP-1 and MMP-3 expression and secretion in HDFs

UV irradiation of cultured HDFs *in vitro* or human skin *in vivo* induces expression of MMP-1 and MMP-3, which play important roles degrading ECM components during skin ageing (13–16). Varani *et al.* (17) reported that MMP levels increase and collagen synthesis decreases with age in sun-protected human skin *in vivo*. In this study, we examined whether DHA_vD inhibited UVB-induced MMP-1 and MMP-3 expression. Analysis by Western blot revealed that UVB irradiation increased MMP-1 and MMP-3 protein levels in HDFs and that DHA_vD inhibited UVB-induced upregulation of MMP-1 and MMP-3 (Fig. 1a). We also determined the effect of DHA_vD on UVB-induced MMP secretion by ELISA. UVB irradiation of HDFs resulted in an increase in MMP-1 and MMP-3 secretion, and DHA_vD significantly diminished UVB-induced MMP-1 and MMP-3 secretion (Fig. 1b). These

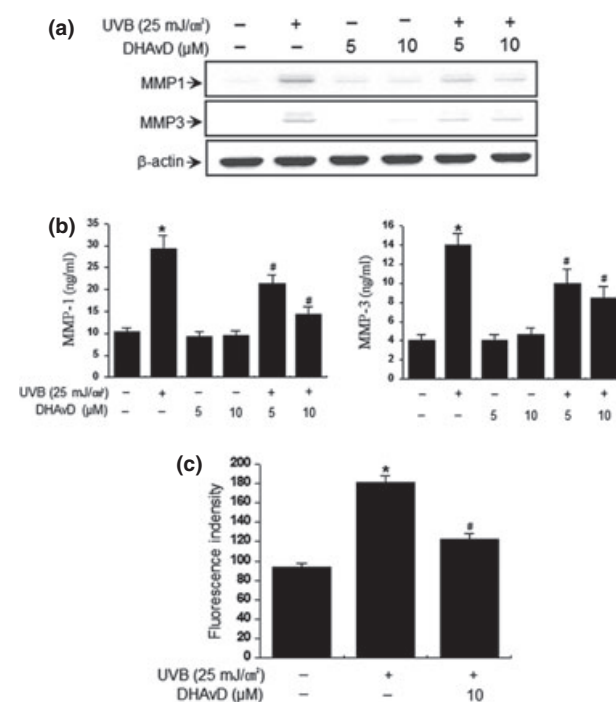


Figure 1. Effects of dihydroavenanthramide D (DHA_vD) on UVB-induced matrix metalloproteinase (MMP)-1, MMP-3 expression and intracellular reactive oxygen species (ROS) in human dermal fibroblasts (HDFs). Cells were stimulated using UVB (25 mJ/cm²) and the indicated concentrations of DHA_vD for 24 h. Cell lysates were analysed by Western blot analysis with anti-MMP-1 and MMP-3 (a). The presence of MMP-1 and MMP-3 in cell-free culture supernatants was measured using a commercially available enzyme-linked immunosorbent assay kit (b). The oxidation-sensitive fluorescent probe DCF-DA was used to analyse intracellular ROS levels. To investigate the effect of DHA_vD on UVB-induced ROS production, HDF cells were exposed to UVB irradiation and then incubated in the presence of DHA_vD for 30 min. Cells were washed twice with PBS and incubated with DCF-DA (10 μM) for 30 min according to the manufacturer's instructions. DCF fluorescence was detected using a FACStar flow cytometer (c). Each value represents the mean \pm standard error of three independent experiments. **P* < 0.01 vs. untreated control; #*P* < 0.01 vs. UVB.

results indicate that DHAvD inhibits the UVB-induced expression and secretion of MMP-1 and MMP-3 in HDFs.

DHAvD inhibits UVB-induced ROS generation in HDFs

UV irradiation induces the oxidative processes involved in skin photoageing. Previous studies have examined the generation of ROS following UVB irradiation, leading to the induction of MMP-1 and MMP-3 (14). Intracellular ROS levels were measured by the DCF-DA fluorescent method to determine whether DHAvD functions as a scavenger for UVB-induced ROS. Our results show that the level of ROS in UVB-irradiated cells increased by approximately 1.9-fold when compared with non-irradiated cells. Pretreating cells with DHAvD in culture medium for 24 h decreased fluorescence by approximately 1.3-fold (Fig. 1c). These results indicate that DHAvD significantly inhibited UVB-induced ROS generation in HDFs.

Effect of DHAvD on UVB-induced NF- κ B and AP-1 DNA-binding activity

The MMP promoter contains NF- κ B and AP-1 binding sites, both of which are centrally involved in MMP gene induction by UVB. To further explore the downstream effects of inhibiting the MAPK pathway by DHAvD in UVB-irradiated HDFs, the DNA-binding activity of NF- κ B and AP-1 was examined by EMSA. DHAvD diminished the UVB-induced nuclear translocation and binding activity of NF- κ B and AP-1 (Fig. 2). Additionally, we confirmed that the increased translocation of p65 and p50 and I κ B α degradation/phosphorylation, and phosphorylation of c-Jun increased as a result of UVB irradiation were significantly suppressed by treatment with DHAvD (Fig. S1). Furthermore, MAPKs are known to upregulate the transcription of genes via UVB-induced ROS generation (18). Therefore, we investigated the effect of DHAvD on MAPK signalling pathways. The UVB-induced phosphorylation of ERK, JNK and p38 was detected, and DHAvD attenuated phosphorylated p38, ERK, JNK and MAPK (Fig. S2). Accordingly, these results suggest that inhibiting p38, ERK, JNK and MAPK signalling by DHAvD might attenuate UVB-induced NF- κ B and AP-1 activation.

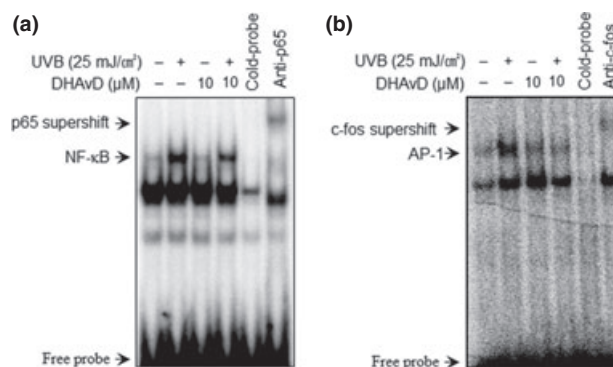


Figure 2. Dihydroavenanthramide D (DHAvD) blocks UVB-induced nuclear factor (NF)- κ B and activating protein (AP)-1 activation in human dermal fibroblasts (HDFs). Cells were stimulated with UVB (25 mJ/cm²) and the indicated concentrations of DHAvD. Following 3-h incubation, nuclear extracts were prepared. NF- κ B (a) and AP-1 (b) DNA binding was analysed using EMSA.

Conclusion

In conclusion, our results provide evidence that DHAvD has an anti-photoageing effect *in vitro*. The DHAvD mechanism may be mediated by inhibiting MMP-1 and MMP-3 expression by inhibiting UVB-irradiated ROS generation. The regulation of MMP expression is possibly controlled by suppressing activation of the MAPK/NF- κ B/AP-1 signalling pathways. Therefore, DHAVD may be a useful candidate for preventing UV light-induced skin photoageing.

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Author contributions

BMH and EMN performed the research. KBK and YOU made the research design. JMK, MSK, WL, JHL and JKH prepared the essential reagents. JSK and YRL made the research design and wrote the paper.

Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Dihydroavenanthramide D (DHAvD) blocks UVB-induced nuclear factor (NF)- κ B and activating protein (AP)-1 signal proteins in human dermal fibroblasts (HDFs). The cells were stimulated with UVB (25 mJ/cm²) and the indicated concentrations of DHAvD. After 3-h incubation, nuclear extracts were prepared. Western blotting was performed to determine the nuclear levels of NF- κ B (p50 and p65) subunits and AP-1 (p-c-Jun) subunits (A), as well as the cytoplasmic levels of p-IKK α / β , IKK α , IKK β and p-I κ B α (B).

Figure S2. Dihydroavenanthramide D (DHAvD) blocks UVB-induced mitogen-activated protein kinase signalling activation in human dermal fibroblasts (HDFs). Cells were stimulated with UVB (25 mJ/cm²) and then treated for 15 min in the presence or absence of DHAvD. Cell lysates were prepared for Western blotting with specific p-p38, p38, p-JUN-N terminal kinase, JNK, p-extracellular regulated kinase (ERK) and ERK antibodies.