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Phloroglucinol enhances the repair of UVB radiation-induced DNA damage *via* promotion of the nucleotide excision repair system *in vitro* and *in vivo*

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ABSTRACT

Exposure to solar UVB radiation can lead to the formation of DNA lesions such as cyclobutane pyrimidine dimers (CPDs). Nucleotide excision repair (NER) is critical for the repair of CPDs induced by UV radiation. The purpose of this study was to investigate the ability of phloroglucinol to protect against the formation of UVB-induced CPDs *in vitro* and *in vivo*. Exposure to UVB radiation increased the number of CPDs in both HaCaT keratinocytes and mouse skin; however, these increases were reduced by treatment with phloroglucinol. Expression levels of xeroderma pigmentosum complementation group C (XPC) and excision repair cross-complementation 1 (ERCC1), which are essential components of the NER pathway, were reduced following UVB exposure, although phloroglucinol treatment recovered these levels in both HaCaT keratinocytes and mouse skin. Phloroglucinol also inhibited UVB-induced reductions in binding of the transcription factors specificity protein 1 to the XPC promoter. These results demonstrate that phloroglucinol can protect cells against UVB-induced DNA damage by inducing NER.

1. Introduction

Skin cancers are the most common malignancies in light-skinned populations, and worldwide skin cancer incidences are continuing to increase [1]. Exposure to ultraviolet B (UVB) radiation (range, 280–320 nm) is a well-established epidemiologic risk factor for skin cancer [2] and UVB-induced DNA damage is a major molecular mechanism responsible for the development of this disease [3]. The major types of DNA damage induced by UVB in the skin are DNA photoproducts, including cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts [4]. CPDs give rise to C to T and CC to TT transition mutations and have been recognized as a molecular trigger for the initiation of photo-carcinogenesis [5]. Repair of CPDs by DNA repair systems reduces photo-carcinogenesis considerably [6].

Among the various UV-induced DNA repair mechanisms, nucleotide excision repair (NER) represents the essential endogenous repair system [7]. NER proceeds through two distinct but overlapping pathways: transcription-coupled repair, which specifically removes lesions from the transcribed strand of active genes, and global genome repair (GGR), which removes lesions throughout the genome [8]. During GGR, xeroderma pigmentosum complementation group C (XPC) is one of the first proteins to recognize DNA damage. Subsequently, transcription factor II human (TFIIH), which comprises XPB, XPD and several other subunits, unwinds the DNA helix through its DNA helicase activity, most likely in concert with XPG, XPA and replication protein A. Next, along with XPF, excision repair cross-complementation group 1 (ERCC1) excises a short oligonucleotide encompassing the UV-induced lesion and the resulting gap is filled by replicative DNA polymerases that use the complementary strand as a template [8,9]. Defective NER is associated with several human diseases, including XP, which has a high incidence of skin cancers, as well as Cockayne syndrome and trichothiodystrophy [10].

Brown algae including *Ecklonia cava* are rich in the polyphenol compound phloroglucinol (1,3,5-trihydroxybenzene) [11]. Our group demonstrated recently that phloroglucinol attenuates the activity of matrix metalloproteinase-1 in UVB-irradiated human

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HaCaT keratinocytes [12] and protects human keratinocytes against UVB-induced oxidative stress by scavenging reactive oxygen species [13]. However, the protective effects of this compound against UVB-induced DNA damage have not been studied in detail. Therefore, the aim of this study was to investigate the ability of phloroglucinol to protect cells against CPDs induced by UVB.

2. Materials and methods

2.1. Reagents

Phloroglucinol, and the anti-actin antibody were purchased from Sigma–Aldrich (St. Louis, MO, USA). The anti-XPC and anti-TATA-binding protein antibodies were purchased from Abcam (Cambridge, MA, USA). The anti-ERCC1, and anti-specificity protein 1 (SP1) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-cyclobutane pyrimidine dimers (CPDs), clone TDM-2 was purchased from Cosmo Bio Co., LTD (Tokyo, Japan). Anti-thymine dimer-horseradish peroxidase (HRP) mAb, clone KTM53 was purchased from Kamiya Biomedical Company (Seattle, WA, USA). All other chemicals and reagents were of analytical grade.

2.2. UVB source and exposures

Cells were exposed to UVB light at a dose of 30 mJ/cm^2. A CL-1000 M UV crosslinker (UVP, Upland, CA, USA) that delivered a UVB energy spectrum of 280–320 nm was used as the source. For the mouse experiments, a UV lamp (UVP) was used as the source of UVB light and the dose was quantified using an HD2102.2 photoradiometer (Delta Ohm, Caselle di Selvazzano, Italy). The intensity of the light source was 364 ± 3 μW/cm^2. To expose the dorsal skin of each mouse to a dose of 60 mJ/cm^2 daily for 3 days, the UVB exposure time was 172 s.

2.3. Cell culture

Human HaCaT keratinocytes (Amore Pacific, Gyeonggi-do, Republic of Korea) were maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO_2/95% air. The cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B.

2.4. Treatment of animals

Male 7-week-old Balb/c mice (Orient Bio Inc., Gyeonggi-do, Republic of Korea) were used for all experiments. The animals were maintained at a controlled temperature of 25–28 °C under a 12 h/12 h light/dark cycle and were provided with a standard diet and drinking water *ad libitum*. All experimental procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at Jeju National University (Jeju, Republic of Korea) (permission number: 2012-0006). The mice were divided into the following four groups ($n = 5$ mice/group): (i) untreated normal control, (ii) vehicle plus UVB (60 mJ/cm^2), (iii) phloroglucinol (10 mg/ml) plus UVB (60 mJ/cm^2), and (iv) phloroglucinol (50 mg/ml) plus UVB (60 mJ/cm^2). Following each daily UVB exposure, 200 μl of phloroglucinol or phosphate buffered saline (PBS) was applied to the dorsal skin of each. Studies were conducted 24 h after the last UVB exposure.

2.5. Enzyme-linked immunosorbent assay (ELISA) and dot blot analyses for CPDs detection

Genomic DNA was purified using the Wizard® genomic DNA purification kit (Promega, Madison, WI, USA). The concentration of DNA was measured using a Qubit fluorometer (Invitrogen, Eugene, OR, USA) and the Quant-iT^{TM} dsDNA HS assay kit (Invitrogen). An ELISA was used to determine the quantities of CPDs, as described by Mori et al. [14]. Briefly, 96-well cell culture plates pre-coated with 0.003% protamine sulfate were incubated with 10 ng of purified genomic DNA in PBS at 37 °C overnight. The TDM-2 antibody (Cosmo Bio Co., Ltd. Tokyo, Japan) was used for detection. After subsequent incubation with a Biotin-conjugated F(ab′)2 fragment of anti-mouse IgG (Molecular Probes Inc., Eugene, OR, USA) and peroxidase-conjugated streptavidin (Invitrogen), the optical density of the substrate solution, which comprised 8 mg of o-phenylene diamine in 4 μl of 35% H_2O_2 and 20 ml citrate-phosphate buffer (pH 5.0), was measured at 492 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). For dot blot analysis, 500 ng of heat-denatured DNA were dotted in triplicate onto a positively charged nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) that was pre-wetted in 6× saline-sodium citrate buffer. After blotting, the dots were rinsed twice with 100 μl of PBS. The membranes were blocked by incubating overnight at 4 °C in PBS containing 5% non-fat dry milk and 0.1% Tween 20, and then incubated for 1 h at 37 °C with an anti-thymine dimer-HRP antibody. After extensive washing with PBS containing 0.5% non-fat dry milk and 0.1% Tween 20, the membranes were incubated for 1 h at room temperature with a 1:2,000 dilution of a secondary anti-mouse HRP-conjugated antibody in PBS-TPBS buffer containing 0.5% non-fat dry milk and 0.1% Tween 20. The blots were then washed extensively with the same buffer lacking the antibody, and peroxidase activity was determined using the enhanced chemiluminescence blotting detection system.

2.6. Immunofluorescence microscopy for CPDs detection

The cells were seeded onto a 4-well chamber slide at a density of 2×10^5 cells/ml, treated with 10 μM phloroglucinol, and then exposed to UVB radiation 1 h later. After incubation for 24 h at 37 °C, the cells were fixed for 10 min with 4% formalin in PBS, and then permeabilized by incubating with 0.5% Triton X-100 in PBS for 5 min on ice. To denature the cellular DNA, 2 M HCl was added to each well for 30 min. To prevent non-specific binding, the cells were incubated with 20% FBS in PBS for 30 min at 37 °C. Subsequently, the cells were incubated for 30 min at 37 °C with 200 μl of the anti-TDM-2 antibody diluted in PBS containing 5% FBS. Next, 200 μl of an Alexa Fluor 594-conjugated F(ab′)2 fragment of anti-mouse IgG (H + L) diluted 1:100 in PBS containing 5% FBS was added to each well and the cells were incubated at 37 °C for 30 min with shaking. After washing with PBS, the stained cells were mounted onto microscope slides in mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). Images were collected using the LSM 510 program on a Zeiss confocal microscope.

For immunohistofluorescence analysis, mouse skin tissue sections were deparaffinized and rehydrated in xylene, followed by a series of decreasing concentrations of ethanol. The rehydrated tissue sections were incubated with a 0.3% methanol–H_2O_2 solution for 30 min to block endogenous peroxidase activity, and then incubated overnight at 4 °C with a primary antibody against a thymine dimer. The experimental procedures that followed were as described for immunocytochemistry.