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Original

Ultraviolet Action Spectrum and Effect of EPC-K1 on Ultraviolet Radiation-induced Injury in Cultured Normal Human Epidermal Keratinocytes

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Abstract : This study was aimed to determine the ultraviolet (UV: 235–310 nm) action spectrum for killing normal human epidermal keratinocytes (NHEK) and to investigate the preventive effect of EPC-K1, a phosphate diester of vitamin C and vitamin E on UV radiation-induced NHEK injury. NHEK were cultured in EpiLife® medium supplemented with Human Keratinocyte Growth Supplement Kit. NHEK viability was determined by crystal violet (CV) staining 48 h after the UV irradiation. The mRNA expressions of the C/EBP homologous protein (Chop) transcription factor and endoplasmic reticulum-resident molecular chaperone, Bip, were determined by RT-PCR analyses. UV was especially effective in killing NHEK when applied in the wavelength region of 250–280 nm. The minimum exposure dose required to kill 50% of cells (LD_{50}) was 1.64 mJ/cm^2 at 269 nm. At 235 and 310 nm, the LD_{50} for NHEK was 6.62 and 293 mJ/cm^2, respectively. Irradiation of 660-mJ/cm^2 at 310 nm significantly decreased the cell viability to 30% of control (without irradiation). The addition of 0.1 mM EPC-K1 after irradiation returned the cell viability to 118%. Six hours after the 660-mJ/cm^2 irradiation at 310 nm, Chop and Bip mRNA levels in NHEK were increased to 487% and 283%, respectively, and were not significantly affected by EPC-K1. Chop and Bip are responsive to ER stress. These results suggested that EPC-K1 exerts a protective effect against UV-induced NHEK injury, and further studies should investigate the molecular mechanism underlying this effect.

Key words : normal human epidermal keratinocytes, ultraviolet irradiation, EPC-K1, endoplasmic reticulum stress

Introduction

Human skin routinely undergoes damage from sunlight and other sources of environmental irradiation. Ultraviolet (UV) irradiation has been identified as a cause of several adverse cutaneous effects such as sunburn[1)], photoaging[2)], and skin cancer[3)]. All of these conditions are closely correlated with an increase in photo-oxidative stress[4–7)]. In sunburn

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following excessive exposure to solar UV radiation, epidermal keratinocyte death occurs primarily by apoptosis[2, 8]. UV irradiation induces reactive oxygen species (ROS) production and subsequent apoptotic cell death in keratinocytes[10]. Apoptosis is also associated with endoplasmic reticulum (ER) stress through ROS generation[11]. In contrast, narrow-band UVB (311 ± 2 nm) irradiation is used to treat psoriasis vulgris[12], vitiligo[13], mycosis fungoides[14], and atopic dermatitis[15], although excessive narrow-band UVB is a common cause of skin inflammation and it might increase the risk of carcinogenesis. Therefore, antioxidant protection might be useful to protect against cutaneous injury from UV irradiation.

A phosphate diester of vitamin C and vitamin E, EPC-K1, is a scavenger of both hydrophilic and hydrophobic radicals[16], it inhibits lipid peroxide production in retinal homogenates[17], and inhibits phospholipase A_2 activity[18]. Epigallocatechin gallate (EGCG) is a potent antioxidant and shows photochemopreventive effects in several *in vitro* and *in vivo* systems[19–21]. From our comparative study of antioxidants on iron-induced peroxidation in bovine retina, EPC-K1 and EGCG were quite active with IC_{50} values of 20 μM and 6.8 μM, respectively[17]. This study investigated the preventive effect of EPC-K1 and EGCG on UV radiation-induced injury to normal human epidermal keratinocytes (NHEK) by measuring effects on C/EBP-homologous protein (CHOP), a transcription factor, and the chaperone HSP70 family member, BIP, as indicators of ER stress[22]. CHOP participates in adaptive responses of the epidermis following UV exposure *in vivo*[23].

Additionally, we determined the UV (235–310 nm) action spectra for killing NHEK because UV radiation of different wavelengths has different effectiveness in eliciting NHEK injury.

Methods

Cell culture

NHEK were obtained from Lonza (Walkersville, MD, USA). NHEK were cultured in EpiLife® medium (Cascade Biologics, Portland, OR, USA) supplemented with human keratinocyte growth supplement (HKGS Kit ; Cascade Biologics) containing bovine pituitary extract, bovine insulin, hydrocortisone, bovine transferrin, human epidermal growth factor, gentamicin, and amphotericin. The cells were grown in 75-cm^2 plastic flasks at 37°C in a humidified atmosphere containing 5% CO_2. The medium was changed every 2–3 days. The cells were harvested at 80% confluence and transferred using a trypsin neutralizing solution (Lonza, Walkersville, MD, USA). Cell passages 4 or 5 were used for this study.

UV exposure

A xenon-lamp light source (MAX-301, Asahi Spectra Co Ltd, Tokyo, Japan) was used for UV exposure. This apparatus is equipped with band pass filters that isolate specific wavelength regions of UV radiation. The UV output was delivered to the cells with a uniform irradiance by a quartz light guide (Asahi Spectra) and a quartz collimating lens (Asahi

Spectra).

Before exposure at each wavelength, irradiance was measured at the position of the target cells with a radiometer (IL 1400A, International Light Technology, Peabody, MA, USA) connected to a silicon-photodiode detector (SEL033, International Light). Exposure durations were determined by dividing doses to be achieved by the measured irradiance. After exposure, irradiance was remeasured, averaged with the first measurement, and then used to calculate the accurate exposure dose. The radiometer was calibrated prior to each experiment.

Before exposure for subconfluent NHEK, the medium was replaced with phosphate-buffered saline (PBS), which is free of any photoactive compounds. After irradiation, the PBS was immediately replaced with EpiLife® medium.

Determination of cell viability : crystal violet (CV) staining

NHEK were cultured in 96-well plates to 80% confluence before the exposure to UV irradiation. After 48 h of UV irradiation, the medium was removed from each well, and then the plates were washed with saline and lightly dried. Cells were then stained by incubation at room temperature for 15 min with 50 μl/well of 0.1% crystal violet (CV; Wako Pure Chemical Industries Ltd, Osaka, Japan) and 1% methanol in PBS. Plates were then washed in PBS and dried. After adding sodium dodecyl sulfate (Wako Pure Chemicals Industries Inc, Hercules, CA, USA) to the wells, their absorbance at 570 nm, which is expected to be proportional to the number of living cells, was measured using a microplate reader (Model 680 XR, Bio-Rad Laboratories). Cell viability was calculated from the absorbance by linear interpolation between the mean absorbance readings for the negative and positive controls, which correspond to cell viabilities of 0% and 100%, respectively.

Determination of the UV action spectrum for killing NHEK

NHEK in 96-well plates with PBS were exposed to narrow-band UV radiation with a bandwidth of approximately 10 nm at 10 different wavelengths from 235 nm to 310 nm (Table 2). After the irradiation, NHEK were cultured for 24 h, and then the LDH activity was measured. After 48 h of culturing, the cell viability was determined by CV staining. The exposure dose required to kill 50% of cells (LD_{50}) was derived from the cell viability for each wavelength and plotted against wavelength to obtain action spectra.

Measurement of the effectiveness in antioxidants on 310 nm-induced injury in NHEK

NHEK in 96- or 6-well plates with PBS was irradiated by 660 mJ/cm^2 at 310 nm. After irradiation, the PBS was immediately replaced with EpiLife® medium and 0.1 mM L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12 -trimethyltridecyl)-2H-l-benzopyran-6yl-hydrogen phosphate] potassium salt (EPC-K1, Senju Pharmaceutical, Osaka, Japan) or 0.1 mM epigallocatechin gallate (EGCG, Sigma Aldrich, St. Louis, MO, USA). After a further 6 h,

Table 1. The sequences of the primers

	Sequences of oligonuclectide primers		PCR product(bp)	Genebank-accession number
Bip	Sense	5'-CGTGTTCAAGAACGGCCG-3'	381	M19645
	Antisense	5'-CGTAGACAGTACGACAGCAACTGT-3'		
Chop	Sense	5'-GGCAGCTGAGTCATTGCC-3'	496	X71427
	Antisense	5'-GCAGATTCACCATTCGGTCA-3'		
β-actin	Sense	5'-TTGTAACCAACTGGGACGATATGG-3'	292	X00351
	Antisense	5'-GATCTTGATCTTCATGGTGCTAGG-3'		

Table 2. The exposure dose required to kill 50% of NHEK (LD_{50}) at each wavelength.

Wavelength (nm)	235	242	250	261	269	280	292	300	306	310
CV staining	7.03	3.63	2.58	1.68	1.64	2.46	4.42	16.44	70.15	292.58

LD_{50} values show in mJ/cm^2.

NHEK in the 6-well plates were collected for RT-PCR analysis. The cell viability of NHEK was determined by CV staining at 48 h after the treatment.

RT-PCR analysis

After 6 h of 660 mJ/cm^2 irradiation at 310 nm, NHEK in 6-well plates were collected and stored at −40°C until used for assay. Chop (C/EBP homologous protein) and Bip (the chaperone HSP70 family mRNA in NHEK) were analyzed by RT-PCR at 6 h after the 310-nm exposure as markers of endoplasmic reticulum (ER) stress. Total RNA was extracted from NHEK using the QIAamp RNA Mini kit (QIAGEN K.K, Tokyo, Japan). RT-PCR analysis was carried out using the Omniscript RT Kit (QIAGEN) according to the manufacturer's instructions. Table 1 details the primers used for amplification. The PCR products were electrophoresed through a 2.0% agarose gel visualized by ultraviolet transillumination (Atto Corp, Tokyo, Japan). Quantification was performed by densitometry using a scanner and Scion Image Version 4.02 software. The ratio of target cDNA to β-actin was used as a relative estimate of mRNA abundance. Each mRNA expression is shown as the percentage of expression in the control NHEK.

Statistical analysis

Data were expressed as mean ± SE. Statistical significance was assessed by Student's t-test. *P* values less than 0.05 were considered significant.

Results

The UV action spectrum for killing NHEK

At each exposure wavelength, cell viability assessed by CV staining decreased with

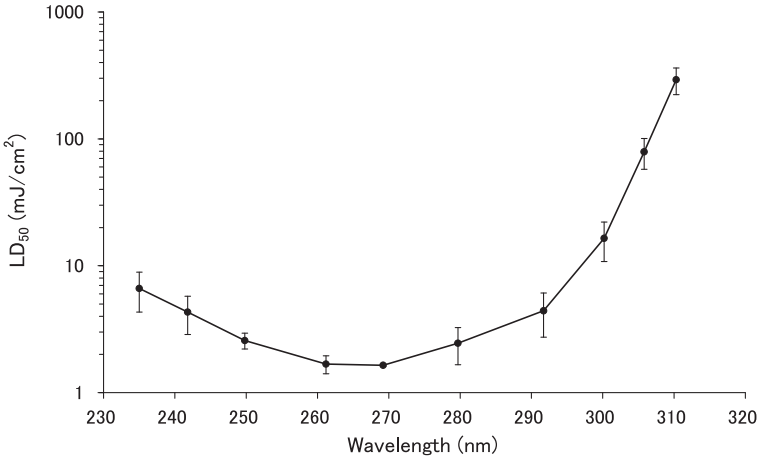

Fig. 1. The ordinate shows the exposure dose required to kill 50% of cells (LD_{50}, mJ/cm^2). The abscissa shows wavelength (nm). LD_{50} was derived from the cell viability. Data show the average±SD for 3–6 independent experiments.

increasing radiant exposure in a manner described by a cumulative log normal distribution function. The LD_{50} values were obtained from this function (Table 2). Action spectra were constructed by plotting the LD_{50} against wavelength (Fig. 1). They have a broad minimum in the approximate range of 250–280 nm, showing that UV is the most hazardous in this range, rising steeply toward both longer and shorter wavelengths.

Effect of EPC-K1 and EGCG on 310 nm-induced NHEK viability

The cell viability was decreased to 30% of control NHEK (no exposure) after 48 h of 310-nm irradiation at 660 mJ/cm^2. Following the addition of 0.1 mM EPC-K1 and 0.1 mM EGCG, the cell viabilities significantly increased to 117% (n = 12) and 110% (n = 8) of control activity, respectively (Fig. 2). Thus, EPC-K1 at 0.1 mM and 0.1 mM EGCG did not influence control cell viability.

Effect of EPC-K1 and EGCG on 310 nm-induced ER stress in NHEK

The Chop mRNA/β-actin mRNA ratio in control NHEK was 0.24 ± 0.05 (n = 7). At 6 h after 660 mJ/cm^2 of 310-nm irradiation, the Chop mRNA/β-actin mRNA ratio increased to 1.19 ± 0.28 (487% of control, n = 7). Following the addition of 0.1 mM EPC-K1 and 0.1 mM EGCG after irradiation, Chop mRNA/β-actin mRNA ratios were 1.00 ± 0.1 and 1.31 ± 0.05, respectively. When 0.1 mM EPC-K1 and 0.1 mM EGCG were added to the NHEK, the ratios were 0.64 ± 0.08 and 0.91 ± 0.2, respectively (Fig. 3a).

The Bip mRNA/β-actin mRNA ratio in control NHEK was 0.399 ± 0.08 (n = 7), and increased to 1.13 ± 0.16 (283% of control, n = 7) following 660 mJ/cm^2 of 310-nm irradiation. Bip mRNA/β-actin mRNA ratios were not significantly affected by 0.1 mM EPC-K1

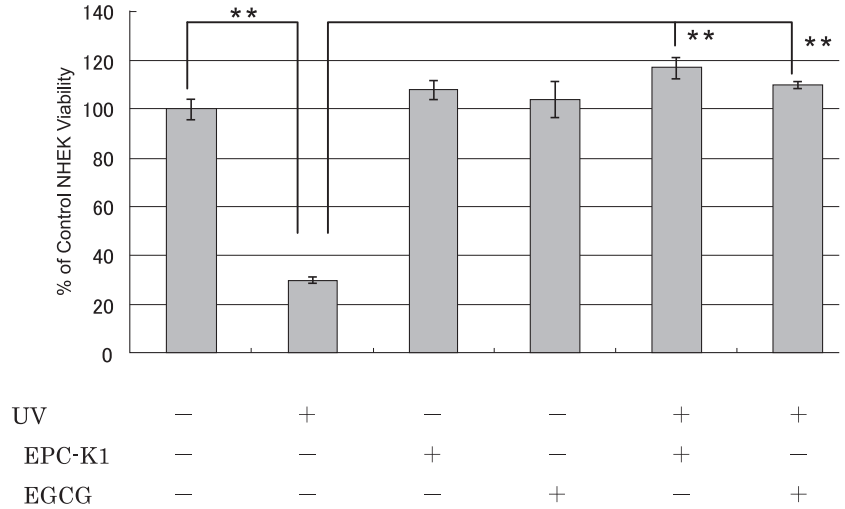

Fig. 2. Comparison between protective effect of EPC-K1 and EGCG addition on 310-nm irradiation of NHEK. After the irradiation (660 mJ/cm^2), 0.1 mM EPC-K1 or 0.1 mM EGCG was added to the medium. After a 48-h incubation, NHEK viability was measured by CV staining. Cont = without irradiation; UV = irradiated untreated cells; EPC-K1 = 0.1 mM EPC-K1 added to cells without irradiation; EGCG = 0.1 mM EGCG added to cells without irradiation. Results are given as a percentage of control values (n = 19) and represent the mean ± SE of seven independent experiments. $^*P < 0.05$, $^{**}P < 0.01$.

and 0.1 mM EGCG addition (0.79 ± 0.11 and 0.68 ± 0.199, respectively). When 0.1 mM EPC-K1 and 0.1 mM EGCG was added to the NHEK, the ratios were 0.56 ± 0.18 and 0.35 ± 0.16, respectively (Fig. 3b).

Discussion

This study of UV (235–310 nm) action spectra for NHEK determined LD_{50} from cell viability by CV staining at each exposure wavelength. The lowest LD_{50} was obtained with 1.64 mJ/cm^2 exposure at 269 nm. UVB (290–320 nm) radiation exposure on mouse skin is directly involved in cyclobutane pyrimidine dimer formation, which was implicated in photocarcinogenesis[24]. The optimal wavelengths for formation of cyclobutane pyrimidine dimers are 270–295 nm[25], thus NHEK death following exposure at 269 nm could be due to DNA damage. UV radiation-induced cell injury results from DNA damage and/or photochemical-induced oxidative stress[25, 26]. Various chromophores contained in human skin could act as endogenous UV sensitizers of photo-oxidative stress[7, 27], by generating ROS that damage DNA and cellular membranes, and promote carcinogenesis[4–7, 28]. UV energy is absorbed by sensitizers following initial formation of an excited state and their subsequent interaction with substrate molecules (type I photochemical reaction) or molecular oxygen (type II photochemical reaction) by energy and/or electron transfer[29]; both resulting in ROS production. Wondrak *et al*[30] reported that extracellular matrix proteins could also act as sensitizers of

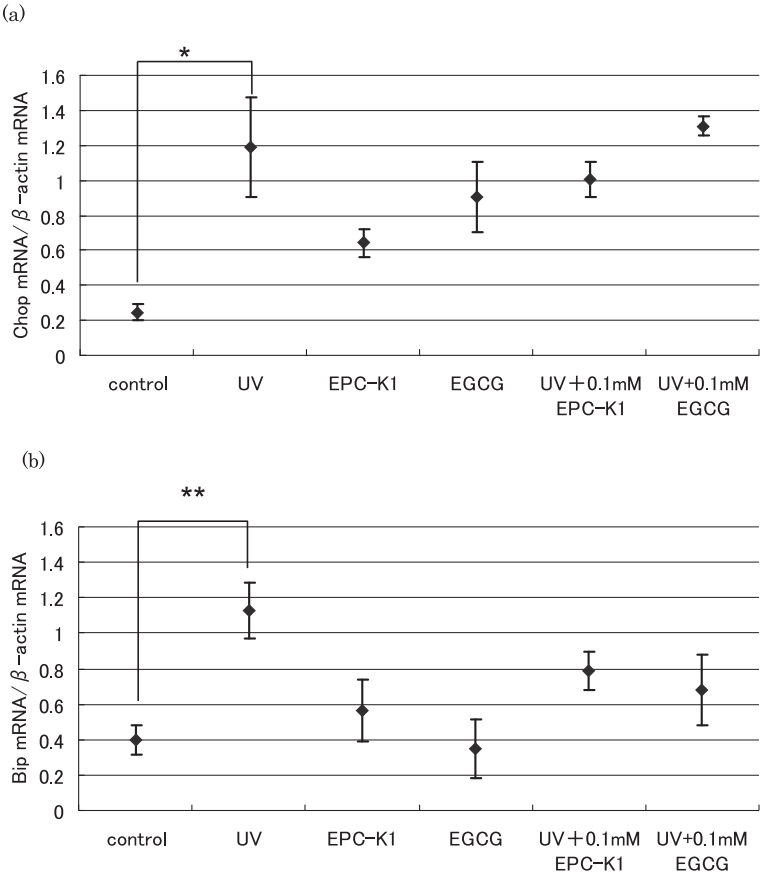

Fig. 3. Effect of EPC-K1 and EGCG on Chop and Bip mRNA in NHEK after 310-nm irradiation. After a 6-h incubation, (a) Chop mRNA and (b) Bip mRNA were measured by RT-PCR. Each value was normalized against β-actin mRNA. Cont = without irradiation; UV = irradiated untreated cells; EPC-K1 = 0.1 mM EPC-K1 added to cells without irradiation; EGCG = 0.1 mM EGCG added to cells without irradiation. Results are given as a percentage of control values (n = 7) and represent the mean ± SE of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

photo-oxidative stress.

ROS production has been detected in cultured human skin cells[31-33], skin homogenates[34], and intact skin[35] following exposure to UV irradiation. ROS activate the receptors in keratinocytes or fibroblasts for growth factors or cytokines. This upregulates AP-1 via activation of transduction signaling pathways, overexpression of matrix metalloproteinase (MMP), and decreased levels of collagen I and III in extracellular matrix, lead to skin photoaging[31].

This study investigated the hazard of 310 nm to NHEK and demonstrated a preventive effect of EPC-K1 and EGCG, which both effectively reduced NHEK death. Bip and CHOP mRNA expressions were significantly increased by 310-nm exposure (Fig. 3). Fol-

lowing ER stress, a series of signaling pathways, referred to as the ER stress response or unfolded protein response (UPR), is activated. Accumulation of unfolded or misfolded proteins are sensed by resident transmembrane sensors and normally held in an inactive state by the binding of intraluminal ER chaperones, especially Bip[36, 37]. Increased CHOP expression is also a hallmark of the ER stress response. Severe or prolonged ER stress induces apoptosis[36, 37]. Sunburn is a cutaneous reaction following excessive exposure to solar UV radiation and the subsequent death of epidermal keratinocytes primarily by apoptosis[2, 8]. EPC-K1 and EGCG did not affect the expressions of Bip and CHOP mRNA in this study.

EGCG is a potent antioxidant and has photochemopreventive effects in several *in vitro* and *in vivo* systems[19-21]. Katiyar *et al*[38] reported that EGCG inhibits UVB (290-320 nm) -induced H_2O_2 production and activation of mitogen-activated protein kinase (MAPK) signaling pathways. EPC-K1 is also suppressed by ROS[16], therefore, the inhibition of 310 nm-induced injury in NHEK by EPC-K1 could involve MAPK signaling. In addition, EPC-K1 suppresses lipid peroxide production[17] and inhibits phospholipase A_2 activity[18]. Future studies should therefore investigate the molecular mechanism underlying the potential photoprotective effect of EPC-K1 on skin.

Skin inflammation from narrow-band UVB exposure is blocked by sunscreen and can be treated by steroidal anti-inflammatory drugs. The simple model system described here could be used to study new-generation therapeutic agents and determine their protective effect from UV irradiation injury of skin.

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