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Protective Effects of EGCG on UVB-Induced Damage in Living Skin Equivalents

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In this study, we evaluate the effects of (-)-epigallocatechin-3-gallate (EGCG) on ultraviolet B (UVB)-irradiated living skin equivalents (LSEs). Histologically, UVB irradiation induced thinning of the LSE epidermis, whereas EGCG treatment led to thickening of the epidermis. Moreover, EGCG treatment protected LSEs against damage and breakdown caused by UVB exposure. Immunohistochemically, UVB-exposed LSEs expressed p53, Fas, and 8-hydroxy-deoxyguanosine (8-OHdG), all of which are associated with apoptosis. However, EGCG treatment reduced the levels of UVB-induced apoptotic markers in the LSEs. In order to determine the signaling pathways induced by UVB, Western blot analysis was performed for both c-Jun NH_2-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), which are associated with UVB-induced oxidative stress. UVB activated JNK in the epidermis and dermis of the LSEs, and EGCG treatment reduced the UVB-induced phosphorylation of JNK. In addition, p38 MAPK was also found to have increased in the UVB-exposed LSEs. Also, EGCG reduced levels of the phosphorylation of UVB-induced p38 MAPK. In conclusion, pretreatment with EGCG protects against UVB irradiation *via* the suppression of JNK and p38 MAPK activation. Our results suggest that EGCG may be useful in the prevention of UVB-induced human skin damage, and LSEs may constitute a potential substitute for animal and human studies.

Key words: EGCG, UVB, Living skin equivalents, Apoptosis, MAPK

INTRODUCTION

Ultraviolet (UV) radiation, particularly UVB (290~320 nm), has been demonstrated to be the pivotal casual factor in human and animal skin cancers (Kraemer, 1997). UVB exposure has been established to induce adverse effects, such as erythema, skin pigmentation, sunburn cell formation, hyperplasia, and photoaging in mammalian skin (Naylor, 1997). Moreover, UVB damages DNA, resulting in mutations of the genes crucial for oncogenesis. It is widely accepted that UVB-induced reactive oxygen species (ROS), such as the superoxide anion, hydroxyl radical, and hydrogen peroxide, are responsible for skin damage after UVB exposure (Nishigori *et al.*, 2004). On the other hand, it has also been shown that a wide range of antioxidants exert protective effects against UVB-induced ROS and photocarcinogenesis (Gensler *et al.*, 1996; Wang *et al.*, 1991). However, excessive ROS may lead to oxidative damages and skin cancer, when its formation exceeds the defense properties of antioxidants.

The skin consists of two main layers, the epidermis and dermis. Because the epidermis and dermis are constantly interacting, the monolayer culture system alone cannot fully account for the complexity of skin biology. Thus, living skin equivalents (LSEs) may constitute a new system for studying skin response, such as skin biogenesis, wound healing, and the regulation of epidermal proliferation and differentiation (Casasco *et al.*, 2001). In addition, LSEs provide a unique method for testing cutaneous toxicity and pharmacological effects, and may be able to replace animal experiments and preliminary clinical studies in this regard (Regnier *et al.*, 1990). For this reason, LSEs constitute a useful model for the study of skin responses

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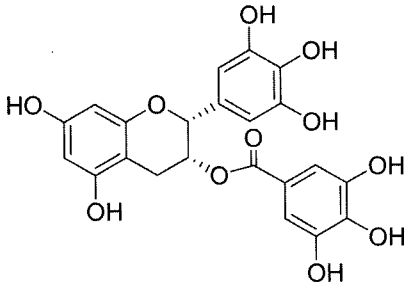

Fig. 1. Chemical structure of (-)-epigallocatechin-3-gallate (Molecular Weight = 458.37)

to UV irradiation.

Green tea is one of the most widely consumed beverages worldwide, and green tea polyphenols have been identified as healthful substances (Yang *et al.*, 2000). The polyphenols in green tea are catechins, a family of compounds which includes (-)-epigallocatechin-3-gallate (EGCG) (Fig. 1), (-)-epigallocatechin, (-)-epicatechin-3-gallate, (-)-epicatechin (Katiyar *et al.*, 2001b). Catechins, which are known to be effective free radical scavengers and potent antioxidants, protect against chemical carcinogenesis and UV-induced oxidative stress (Katiyar *et al.*, 2000; Nanjo *et al.*, 1999). It has been established that the drinking or topical application of EGCG protects against UVB-induced skin carcinogenesis, and inhibits chemical carcinogenesis in various animal organs (Lu *et al.*, 2002). It has also been demonstrated that the oral administration of tea constituents prevents UVB-induced skin tumorigenesis in SKH-1 mice (Wang *et al.*, 1992) and inhibits the growth of UV radiation–induced papillomas in mouse skin (Mittal *et al.*, 2003). Thus, we propose that the generation of ROS by UVB and chemical carcinogens can be mitigated by EGCG.

In order to avoid animal study, we used LSEs to assess the effects of EGCG on UVB-induced damage. This testing system should be simple and useful as an alternative to animal or clinical study. Since the depth of UVB penetration depends on wavelength, it is possible that UVB exerts different effects on the epidermis and dermis of human skin. Thus, we separated the epidermis and dermis, and also attempted to ascertain the pathways by which UVB-induced signaling occurs.

MATERIALS AND METHODS

Materials

EGCG was purchased from Sigma Aldrich (St. Louis, MO, U.S.A.). Antibody recognizing phospho-specific p38 MAPK (Thr180/Tyr182, # 9211S) was purchased from Cell Signaling Technology, Inc. (Beverly, MA, U.S.A.). Phospho-specific JNK1/2 (Thr183/Tyr185, G-7, sc-6254), total (phosphorylated and non-phosphorylated) JNK2 (D-2, sc-7345), total (phosphorylated and non-phosphorylated) p38 MAPK (A-12, sc-7972), actin (I-19, sc-1616) and p53 (DO-1, sc-126) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.); anti-Fas antibody was from Calbiochem (Ab-1, Darmstadt, Germany); and anti-8-hydroxy-deoxyguanosine antibody was purchased from Chemicon (8-OHdG, AB5830, CA, U.S.A.).

Cell cultures

Epidermal keratinocytes and dermal fibroblasts were isolated from human foreskins, obtained during child circumcision. Skin specimens were processed according to the methods described by Rheinwald and Green (Rheinwald and Green, 1975), and modified in our laboratory with thermolysin (Sigma, Sigma Aldrich, St. Louis, MO, U.S.A.). Keratinocytes were cultured in keratinocyte growth medium (KGM, Clonetics, San Diego, CA, U.S.A.). Fibroblasts were isolated and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, U.S.A.), 1 mM sodium pyruvate, 50 mM streptomycin and 50 U/mL penicillin. Cultured primary keratinocytes and fibroblasts were seeded in dishes, then incubated in 5% CO_2 at 37°C.

Reconstruction of living skin equivalents

LSEs were reconstructed using cultured human keratinocytes and fibroblasts. Dermal substitutes were prepared according to the method described by Bell (Bell *et al.*, 1979), with some modifications (Auger *et al.*, 1995). In brief, type I collagen extracts from rat tail tendons were dissolved by stirring in 1/1000 glacial acetic acid at 4°C, for 48 h. Dermal substitutes were constructed by mixing eight volumes of type I collagen with one volume of 10× concentrated DMEM, and one volume of neutralization buffer (0.05 N NaOH, 0.26 mM $NaHCO_3$, and 200 mM HEPES). Three milliliters of this mixture were poured into a 30 mm polycarbonate filter chamber (3.0 μm Millicell; Millipore, Bedford, MA, USA). After 15 minutes of gelation at 37°C, culture medium was added. In this study, the dermal substitute was prepared by adding 5×10^5 fibroblasts (passage number 6) to each millicell. Human keratinocytes (passage number 2) were also seeded, at a density of 2×10^6 cells, onto the dermal substitute, and then cultured in a submerged state for 1 day, then for 12 days at the air-liquid interface. The growth medium consisted of DMEM and Ham's nutrient mixture F12, at a ratio of 3:1, supplemented with 5% FBS, 0.4 μg/mL hydrocortisone, 1 μM isoproterenol, and 5 μg/mL insulin. A low concentration of epidermal growth factor (EGF, 1 ng/mL, Invitrogen, Carlsbad, CA, USA) was added during the submerged stage of the culture and a higher concentration of EGF (10 ng/mL) was added during the air-liquid interface

phase of the culture. The medium was changed three times per week. Three independent experiments were performed with keratinocytes and fibroblasts obtained from different donors.

EGCG treatment and UV radiation

EGCG (1 μM) was supplemented for 3 days during the air-liquid interface phase and the LSEs were subsequently exposed to 300 mJ/cm^2 UVB with a UVB source (BLE-1T158, Spectronics corp., Westbury, NY, USA). A Kodacel filter (TA401/407. Kodak, Rochester, NY, USA) was used in order to remove light wavelengths of less than 290 nm (UVC). The applied energy was measured with a Waldmann UV meter (model No. 585100; Waldmann Co., VS-Schwenningen, Germany). After UVB irradiation, the LSEs were cultured for a further 24 h at 37°C in 5% CO_2.

Western blot analysis

Twenty-four hours later, cultured specimens were removed from the millicell. The LSEs were trimmed, and the epidermis and dermis were separated. Isolated specimens were lysed in a lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitors (Roche, Manheim, Germany), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA. The lysates were homogenized with a homogenizer, and clarified by 10 minutes of centrifugation at 12,000×g and 4°C. The supernatants were used for a protein assay, performed *via* the Bradford method (Bio-Rad, Hercules, CA, U.S.A.). The soluble fraction was subjected to SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gel. Twenty micrograms of the soluble fraction was loaded per lane. The separated proteins were then transferred onto a PVDF membrane (Imobilon, Millipore, Bedford, MA, U.S.A.). The membranes were saturated with 5% dry milk in Tris-buffered saline, containing 0.4% Tween 20. An immuno-blotting analysis was performed with phospho-specific p38 MAPK, total p38 MAPK, phospho-specific JNK1/2 and total JNK2 antibody overnight at 4°C, and then the specimens were further incubated with horseradish peroxidase-conjugated goat anti-mouse antibody and goat anti-rabbit antibody, respectively. (Zymed Laboratories Inc., South San Francisco, CA, U.S.A.). The p38 and JNK were visualized with an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech, Princeton, NJ, U.S.A.).

Histology and Immunohistochemistry

After 13 days, the LSEs were fixed in Carnoy's solution (ethanol/chloroform/acetic acid 6:3:1) for 30 minutes, and processed for conventional paraffin embedding. For morphological observation, sections were stained with hematoxylin-eosin (H&E), and used for immunohistochemical analysis. Five μm-thick paraffin-embedded sections were used to perform assays for 8-hydroxy-deoxyguanosine (8-OHdG), p53, and Fas. Slides were deparaffinized in xylene, and dehydrated in a graded ethanol concentration series. The sections were then treated with proteinase K (10 μg/mL) or citrate buffer with microwave treatment (pH 6.0, DAKO, Glostrup, Denmark), respectively. The sections were also treated with 3% H_2O_2/PBS solution for 30 minutes at room temperature (RT), in order to halt any endogenous peroxidase activity. Then, the sections were blocked for 30 minutes with DAKO® protein block serum at RT, and incubated overnight at 4°C, with anti-p53 monoclonal antibodies, anti-Fas polyclonal antibodies, and anti-8-OHdG polyclonal antibodies.

Staining was performed using the avidin-biotin-peroxidase-complex technique (DAKO, Glostrup, Denmark), followed by diaminobenzidine, which was used as the chromogen (DAB kit, DAKO), and counterstained with Harris hematoxylin (Sigma). Epidermal thickness was measured using an Olysia® Soft Imaging System (Olympus, Tokyo, Japan).

Statistics

Differences between results were assessed for significance using Student's *t*-test.

RESULTS

EGCG reduces UVB-induced DNA damage in LSEs

UVB radiation is known to penetrate the skin, resulting in damage to the DNA. It triggers DNA base mutations, and forms oxidized macromolecules, notably lipid peroxides. In particular, 8-hydroxy-deoxyguanosine (8-OHdG) is a modified base which is associated with oxidative DNA damage. Thus, 8-OHdG serves as a biomarker for oxidative damage in cellular DNA. In order to examine DNA damage caused by UVB, we observed the pattern of 8-OHdG expression occurring in the LSEs (Fig. 2). UVB exposure resulted in dramatic increases in 8-OHdG expression. However, EGCG treatment diminished this UVB-induced 8-OHdG accumulation (Fig. 2). These data indicate that EGCG pretreatment inhibits UVB-induced damage.

EGCG inhibits UVB-induced apoptosis in LSEs

In order to determine whether UVB-induced DNA-damage is related to apoptosis, we examined the tumor suppressor protein, p53, and the death receptor, Fas, which are both deeply involved in the apoptotic process. We found that UVB induced an increase in the expression of p53 (Fig. 3A). However, EGCG treatment abolished

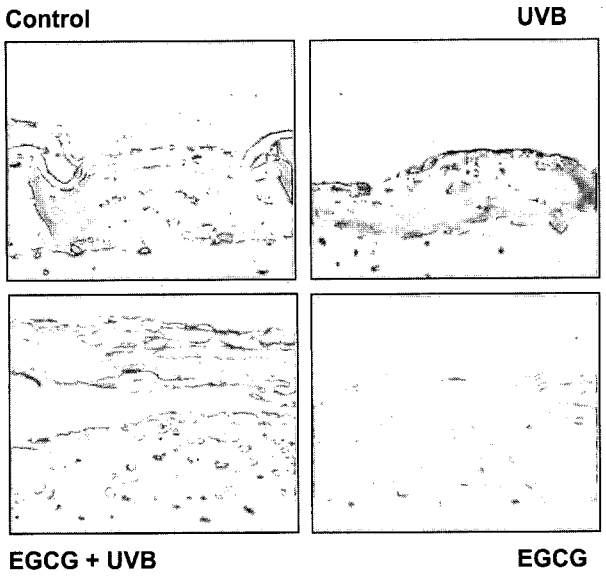

Fig. 2. Inhibition of UVB-induced DNA damage by EGCG treatment in LSEs. LSEs were exposed to 300 mJ/cm^2 of UVB in either the absence or presence of EGCG (1 μM). After 24 h, immunohistochemical detection of 8-OHdG expression was performed, using anti-8-OHdG polyclonal antibodies, and the avidin-biotin-peroxidase-complex technique, as described in "Materials and Methods." Control LSEs were sham-irradiated. UVB-exposed LSEs exhibited increased expression of 8-OHdG expressions. EGCG treatment decreased UVB-induced 8-OHdG expression, and EGCG treatment alone had no effect on 8-OHdG expression levels. Original magnification: ×400.

this UVB-induced p53 increase (Fig. 3A). In a manner similar to that observed with p53, Fas expression also increased as a result of UVB exposure (Fig. 3B). And EGCG treatment inhibited UVB-induced Fas expression (Fig. 3B). Therefore, EGCG treatment appears to protect LSEs from UVB-induced apoptosis.

Histologically, shrinkage of the shape and condensation of the cytoplasm, thinning of the epidermis, and premature death of the keratinocytes were observed after UVB irradiation. UVB irradiation induces aggregated sunburn cells, a typical form of apoptosis (Polakowska *et al.*, 1994). Apoptotic cells characteristically undergo morphological alterations, including losses of cell volume and nucleus condensation (Jacobson *et al.*, 1997). Our findings demonstrate that UVB irradiation results in damage to the keratinocytes, and breakdowns in the structure of the LSEs. However, EGCG treatments restored epidermal thickness and rendered the basal layer more compact (Fig. 2 and 3). Thus, our results indicate that EGCG pretreatment inhibits the UVB-induced breakdown of the LSEs (Table I).

EGCG blocks UVB-induced signaling pathways in LSEs

In order to evaluate the protective mechanisms of

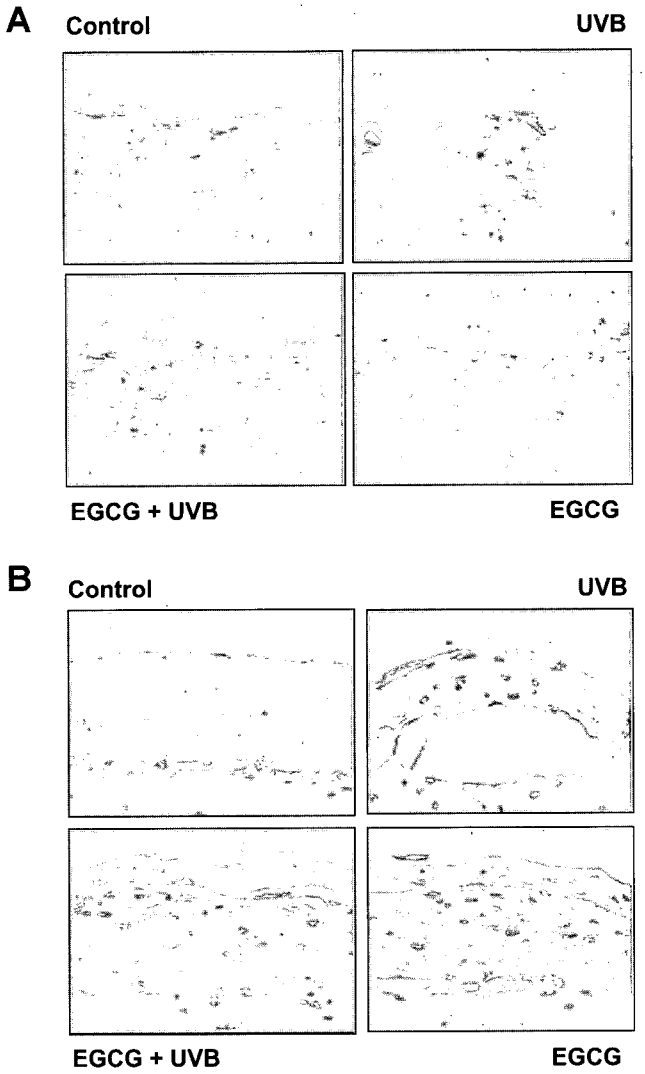

Fig. 3. Inhibition of UVB-induced apoptosis by EGCG treatment in LSEs. LSEs were exposed to 300 mJ/cm^2 of UVB in either the absence or presence of EGCG (1 μM). After 24 h, immunohistochemical detections for p53 (A) and Fas (B) were performed *via* the DAB-peroxidase reaction, as described in "Materials and Method." Control LSEs were sham-irradiated. UVB-exposed LSE exhibited increases in both p53 and Fas expression levels. EGCG treatment decreased the UVB-induced expressions of both p53 and Fas. EGCG treatment alone had no effect on p53 or Fas expression. Original magnification: ×400.

EGCG on UVB-induced apoptosis, we examined UVB-induced signaling pathways. In particular, we were interested in the activation of JNK and p38 MAPK, to help us to determine the effects of UVB on stress-regulated kinases. In the epidermis, UVB increased the phosphorylation of JNK1/2, whereas EGCG treatment suppressed UVB-activated JNK1/2 (Fig. 4A). In addition, p38 MAPK exhibited an activation pattern similar to JNK. EGCG treatment also reduced levels of UVB-activated p38 MAPK. Likewise, in the dermis, UVB activated JNK and

Table I. Epidermal thickness after EGCG and UVB treatment

Experimental group	Epidermis thickness (μm)
Control	87.95 ± 7.9
UVB	58.14 ± 5.3**
EGCG + UVB	83.6 ± 3.1##
EGCG	107.13 ± 3.7**

Epidermal thickness was measured using an Olysia® Soft Imaging System. UVB (300 mJ/cm²)-irradiated LSEs showed thinning of the epidermis. However, EGCG treatment (1 μM) restored epidermal thickness. Data are presented as the means±SD. **P< 0.01 compared to control and ##P< 0.01 compared to UVB-treated LSEs.

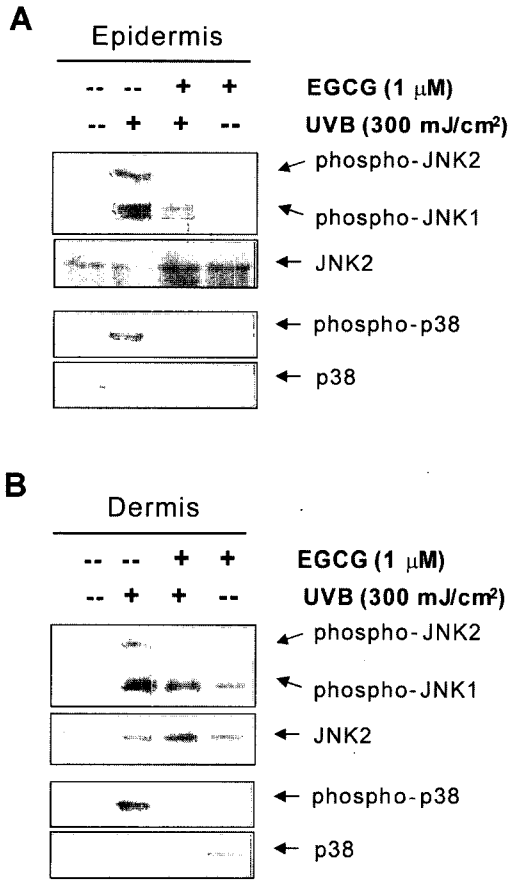

Fig. 4. Inhibition of UVB-induced MAPK activation by EGCG treatment in LSEs. LSEs were treated with EGCG (1 μM) for 3 days before UVB radiation (300 mJ/cm²). Western blot analysis was performed using both the epidermis (A) and the dermis (B) of the LSEs. Blots were incubated with the appropriate primary antibodies at a dilution factor of 1:1000. In the epidermis, UVB induced the activation of JNK1/2 and p38 MAPK, whereas EGCG pretreatment decreased the UVB-induced activation of JNK1/2 and p38 MAPK. Similar results were obtained in the dermis.

p38 MAPK, and EGCG treatment resulted in a drop in the levels of UVB-stimulated stress regulated kinases (Fig. 4B). Thus, EGCG pretreatment inhibits UVB-induced signaling pathways, which are related to apoptosis.

DISCUSSION

LSEs may be considered to be a promising candidate for a substitute for human skin in clinical applications (Casasco *et al.*, 2001; Zacchi *et al.*, 1998). LSEs possess both stratified and cornified layers, and exhibit the same barrier function as actual skin. Hence, we used LSEs as an artificial skin model, to assess the effects of EGCG on UVB-induced skin damage. Our study demonstrates that EGCG treatment ameliorates or reverses UVB-induced breakdown in the structure of the LSEs. It was also discovered that the topical application of EGCG to human skin induces the proliferation of epidermal keratinocytes, resulting in increased epidermal thickness (Chung *et al.*, 2003). Furthermore, EGCG inhibits the UV-induced apoptosis of human epidermal keratinocytes (Chung *et al.*, 2003). These results indicate that EGCG plays an important role in the regulation of epidermal proliferation and, ultimately, survival.

UVB-radiation is able to penetrate skin and induce cellular apoptosis, which manifests itself in several morphological changes, most notably, shrinkage and condensation of the cytoplasm, coupled with nuclear fragmentation (Polakowska *et al.*, 1994). UVB irradiation is also known to induce the generation of ROS and DNA damage (Wei *et al.*, 1999). UVB-generated ROS can induce the activation of stress-regulated signaling pathways, cell membrane damage, and apoptosis (Assefa *et al.*, 1997; Kulms *et al.*, 2002). Thus, antioxidants may exert protective effects with regard to UVB-induced skin damage, by scavenging ROS. 8-OHdG is a representative DNA base-modified product in UVB-induced oxidative stress. ROS-generated 8-OHdG, in turn, induces the transversion of G-C to T-A during DNA replication (Ahmed *et al.*, 1999; Kasai and Nishimura, 1984; Nishigori *et al.*, 1994), and UV-induced carcinoma in mice evidences high 8-OHdG levels (Hattori *et al.*, 1996). Our results indicate that UVB increases the production of 8-OHdG in LSEs, and also that EGCG treatment inhibits the formation of UVB-induced 8-OHdG, bolstering the conclusion that EGCG diminishes UVB-induced DNA-damage.

The tumor suppressor protein, p53, has been detected in a variety of tumor cells, and increases in response to DNA damage and cellular stress signals. It has been demonstrated that p53 is induced by UV irradiation, and subsequently arrests the cell cycle in the G_1 phase. Furthermore, p53 is a crucial factor in apoptotic cell death (Decraene *et al.*, 2001; Kuo and Lin, 2003). In this study, UVB irradiation resulted in increased p53 expression in the LSEs. However, EGCG treatment resulted in a reduction of UVB-induced p53 expression. Apoptosis is a process which is regulated by a number of factors, including the Fas/Fas ligand system (Nagata, 1994). Fas,

an important death-domain receptor, is involved in UVB-induced apoptotic progression. UV irradiation can directly activate Fas, independently of its ligand, thereby inducing a signal cascade which leads to the apoptotic termination of the affected cells (Nagata and Golstein, 1995). Our study demonstrated that exposure to UVB induced Fas expression in the LSEs. However, we also demonstrated that EGCG pretreatment ameliorates this UVB-induced Fas expression. These results suggest that EGCG protects LSEs from apoptosis by reducing UVB-induced p53 and Fas expression.

MAPK pathways play crucial roles in the mediation of extracellular stimuli to intracellular signals, which trigger cellular events including proliferation, differentiation, and apoptosis (Katiyar *et al.*, 2001a). UVB is known to activate both the JNK and p38 MAPK pathways, in a variety of cell types. This study also showed that UVB-exposure activates JNK and p38 MAPK signaling pathways in both the separated epidermis and dermis of the LSEs. However, EGCG treatment resulted in decreases in the JNK and p38 MAPK phosphorylation in both the UVB-exposed epidermis and dermis (Fig. 4). EGCG has been identified as a profound antioxidant. The mechanisms underlying this effect involve modulating cytokines, scavenging of ROS, and the blockage of UV-induced oxidative damage (Wei *et al.*, 1999). EGCG pretreatment thus inhibits UVB-induced oxidative stress, which is associated with JNK and p38 MAPK phosphorylation, following UVB irradiation. It has been reported that p53 stabilization and accumulation are caused by JNK and p38 MAPK phosphorylation in response to oxidative damage (Buschmann *et al.*, 2001; Decraene *et al.*, 2004; Oda *et al.*, 2000). In our study, EGCG was found to inhibit UVB-induced MAPK activation, coupled with a marked down-regulation of UVB-induced p53 protein expression. Collectively, our data indicated that EGCG pretreatment inhibited UVB-induced oxidative stress in the LSEs.

In conclusion, this study demonstrates that EGCG exerts protective effects against UVB irradiation. Thus, EGCG may be quite useful in the treatment of UVB-induced human skin disorders such as aging, carcinoma, and inflammation. Additionally, LSEs may also provide a potential human skin substitute for future clinical study.

ABBREVIATIONS

DMEM, Dulbecco's modified Eagle's medium; EGCG, (-)-epigallocatechin-3-gallate; EGF, epidermal growth factor; JNK, c-Jun NH_2-terminal kinase; KGM, keratinocyte growth medium; LSEs, living skin equivalents; 8-OHdG, 8-hydroxy-deoxyguanosine; p38 MAPK, p38 mitogen-activated protein kinase; ROS, reactive oxygen species; UVB, ultraviolet B.

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