(+)-Catechin prevents ultraviolet B-induced human keratinocyte death via inhibition of JNK phosphorylation

Wen-Bin Wu a, Han-Sun Chiang a, Jia-You Fang b, Shao-Kuan Chen c, Chieh-Chen Huang a,d, Chi-Feng Hung a,*

a School of Medicine, Fu-Jen Catholic University, No. 510, Jhongjheng Rd., Sinjhuang City, Taipei County 24205, Taiwan ROC
b Graduate Institute of Natural Products, Chang Gung University, Kweishan, Taoyuan, Taiwan ROC
c Department of Surgery, Cathay General Hospital, Taipei, Taiwan ROC
d Department of Dermatology, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan ROC

Received 22 October 2005; accepted 25 February 2006

Abstract

High levels of (+)-catechin are found in the skin and seed of many fruits such as apples and grapes. Dietary supplementation with (+)-catechin has been demonstrated to protect epidermal cells against damage induced by ultraviolet B (UVB) radiation. However, the underlying mechanisms are not well understood yet. To determine whether (+)-catechin protects keratinocytes from UVB-induced damage, the viability of UVB- and H2O2-treated cells was determined by cell viability assay. Intracellular H2O2 level was measured by flow cytometry. UVB- or H2O2-induced signaling pathways were detected by Western blotting. The results indicated that (+)-catechin inhibited UVB- and H2O2-induced keratinocyte death. In parallel, intracellular H2O2 generation in keratinocytes irradiated by UVB was inhibited by (+)-catechin in a concentration-dependent manner. (+)-Catechin also inhibited UVB- and H2O2-induced JNK activation in keratinocytes. However, it had little inhibitory effect on UVB- and H2O2-induced ERK and p38 activation even at a higher concentration, suggesting indirectly that JNK activation is required for the induction of apoptosis in keratinocytes exposed to UVB. Finally, we compared the cytotoxicity of (+)-catechin and (-)-epigallocatechin-3-gallate (EGCG) on keratinocytes. Cell viability assay showed that (+)-catechin was relatively nontoxic at higher doses. Taken together, our results demonstrate that (+)-catechin inhibits UVB- and oxidative stress-induced H2O2 production and JNK activation and enhances human keratinocyte survival. However, although it seems that (+)-catechin and EGCG are equally effective in preventing keratinocyte death, (+)-catechin is relatively nontoxic and thus is suitable for developing as an anti-ageing agent for skin care.

© 2006 Elsevier Inc. All rights reserved.

Keywords: (+)-Catechin; Keratinocyte; UVB

Introduction

Ultraviolet (UV) radiation, in particular ultraviolet B (UVB) with a wavelength range between 290 and 320nm, is an important environmental factor because of its hazardous health effects, which include generation of skin cancer (Ananthawamy and Pierceall, 1990), suppression of the immune system (Donawho et al., 1996), and photoaging (Goihman-Yahr, 1996). A growing body of evidence suggests that reactive oxygen species (ROS) are generated by UV radiation cause oxidative damage to cellular components such as mitochondria as well as nuclear DNA damage, which in turn accelerates ageing and contributes to skin cancers (Miyachi, 1995). Among ROS, H2O2 is one of the most important mediators when keratinocytes are irradiated by UVB (Peus et al., 1999a). Many antioxidants have been shown to have protective effects against ROS-induced injury in skin. Antioxidants such as ascorbic acid (Dunham et al., 1982), α-tocopherol (Gensler and Magdaleno, 1991), β-carotene (Mathews-Roth and Krinsky, 1987), selenium (Oxerand et al., 1985) and a mixture of dietary antioxidants (Black and Chan, 1975) have been reported to inhibit UV-induced skin carcinogenesis. (+)-Catechin is a flavanol and a polyphenol. High levels of (+)-catechin are found in the skin and seed of many fruits such as apple and grape. Moreover, red wine and chocolate are...
significant sources of (+)-catechin (Renaud and de Lorgeril, 1992; Arts et al., 1999). However, although (+)-catechin is cheap and abundant in plant sources, little attention has been paid to evaluation of its biological activity in vitro and in vivo systems, possibly due to its lack of a galloyl group. Most past studies focused on gallated catechins, especially (-)-epigallocatechin-3-gallate (EGCG) and (-)-epicatechin-3-gallate (ECG). For instance, EGCG is an effective protectant against the oxidative stress caused by UVB radiation (Katiyar et al., 2001). Topical treatment of human and mouse skin with EGCG before UV exposure significantly reduced UVB-induced erythema development, hydrogen peroxide production and leukocyte infiltration (Hursting et al., 1999; Katiyar and Mukhtar, 2001). In addition, epigallocatechin and ECGG have been shown to protect against UVB-induced skin carcinogenesis in mice (Agarwal et al., 1993; Katiyar et al., 1995). Recently, it was shown that (+)-catechin may have a potential to reduce the risk of UV-induced, oxidative stress-mediated skin diseases (Jeon et al., 2003). The authors demonstrated that dietary supplementation of (+)-catechin can protect mouse skin cells against UVB-induced damage, but its mechanism of action is not fully elucidated.

In the present study, we investigated the effects of (+)-catechin on UVB-induced keratinocyte damage. We provide here the first evidence that (+)-catechin, a cheap and abundant component, available from many sources, can inhibit UVB- and oxidative stress-induced keratinocyte death. The action mechanisms of (+)-catechin are elucidated. These include the inhibition of UVB-induced intracellular hydrogen peroxide (H₂O₂) production and JNK activation.

Materials and methods

Materials

(+)-Catechin, 3-(4,5-dimethylthiazol-2-yl)-2ami,5-diphenyl-tetrazolium bromide (MTT), aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium fluoride (NaF) and sodium orthovanadate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies raised against p38 and p-JNK were from Cell Signaling Technology (Beverly, MA, USA). Antibodies raised against JNK, ERK1/2 and p-p38 were from R&D Systems, Inc. (Minneapolis, MN, USA). Antibody raised against p-ERK1/2 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dihydrorhodamine 123 (DHR 123) was from Molecular Probes (Eugene, OR, USA).

Cell culture

Human immortalized keratinocytes (HaCaT cells) were a gift from Dr. Y.J. Lee in Fu-Jen Catholic University and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (GibcoBRL, Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma Chemical Co.). The cells were cultured in a humidified incubator at 37°C and 5% CO₂. For most experiments, cells reaching a 90%-95% of confluency were starved and synchronized in serum-free DMEM for 24h before further analysis.

Drug treatment and UVB irradiation

Keratinocytes cultured on 1.5-cm culture dish (Costar, Cambridge, MA, USA) were pretreated with various concentrations of (+)-catechin for 2h. After two washes with DMEM, cells were then incubated with 1 ml of phosphate-buffered saline (PBS). UVB irradiation was performed immediately as suggested by the manufacturer. Briefly, cells were irradiated in a Bio-Sun system illuminator from VL (Vilber Lourmat, France) with a UV peak at 312nm. UVB was supplied by a closely spaced array of two UVB lamps, which delivered uniform irradiation at a distance of 10 cm. UVB irradiation dose was 50mJ/cm². After UVB exposure, cells were fed with fresh DMEM in the presence of (+)-catechin, incubated for the indicated time, and collected for further analysis.

Cell viability assays

The viability of cells was determined by MTT and trypan blue exclusion assays. MTT assay has been described previously by our group (Huang et al., 2005). Briefly, PBS- or (+)-catechin-pretreated cells were exposed to UVB or H₂O₂ (2×10⁻³ mol/l) and incubated for an additional 24h. After a brief wash with medium, MTT (0.5 mg/ml in DMEM) was used for the quantification of living metabolically active cells (Mosmann, 1983). Mitochondrial dehydrogenases metabolized MTT to a purple formazan dye, which is measured photometrically at 550nm. Cell viability is proportional to the absorbance measured (Green et al., 1984). For trypan blue exclusion assay, trypan blue-impermeable viable cells were determined by hemocytometer counts under a phase-contrast microscope.

Flow cytometric analysis of intracellular H₂O₂

Intracellular production of H₂O₂ was assayed as previously described (Huang et al., 2005) with minor modification. Briefly, confluent keratinocytes starved with serum-free DMEM were pretreated with various concentrations of (+)-catechin for 2h. Cells were washed with PBS and DMEM, and then treated with DHR 123 (10µg/ml) in DMEM for 30 min. After a brief wash, cells were irradiated by UVB and were collected by scraping and centrifugation. The cell pellets were resuspended in 1 ml of PBS and then analyzed immediately with the Partec CyFlow ML flow cytometer (Partech GmBH, Munster, Germany) at excitation and emission wavelengths of 488 and 525 nm, respectively. Fluorescence signals of 10,000 cells were collected to calculate mean fluorescence intensity of a single cell.

Cell lysate preparation and Western blot analysis of JNK, ERK and p38

Keratinocytes treated with or without UVB were washed with PBS for twice. Cells were lysed in radioimmunoprecipitation
assay buffer [17mM Tris–HCl, pH7.4, 50mM NaCl, 5mM EDTA, 1mM sodium fluoride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1mM sodium orthovanadate, 1mM PMSF, and 1 μg/ml aprotinin and leupeptin (freshly prepared)]. After sonication, the lysate was centrifuged (14,000×g for 10min at 4°C), and supernatant was removed. The protein content was quantified with Pierce protein assay kit (Pierce, Rockford, IL, USA). Total protein was separated by electrophoresis on 10% SDS–polyacrylamide gels and the proteins were electroblotted onto PVDF membranes and then probed using the indicated specific antibodies. Immunoblots were detected by enhanced chemiluminescence (Chemiluminescence Reagent Plus from NEN, Boston, MA, USA). For some experiments, the PVDF membrane was stripped at 60°C for 30min with a stripping buffer (62.5mM Tris–HCl, pH6.7, 2% SDS and 100mM β-mercaptoethanol).

**Statistical analysis**

Otherwise where indicated, data were expressed as mean ± standard errors (S.E.). Comparison of means of two groups of data was made by using the unpaired, two-tailed Student’s t-test. All data were analyzed by SigmaPlot 2000 for Windows (Version 6.00).

**Results**

**(+)-Catechin inhibits UVB-induced keratinocyte death**

To determine the protective effects of (+)-catechin on human keratinocytes, we first performed cell viability assay. In congruence with previous reports (Maalouf et al., 2002; Ahn et al., 2002), MTT assay showed that cell viability of keratinocytes was decreased after UVB exposure (Fig. 1a). However, the decrease was reversed by treatment with (+)-catechin (10 and 100 μM). The result was confirmed by trypan blue exclusion assay. UVB-induced cell death was reduced by 10 and 100 μM of (+)-catechin (Fig. 1b). (+)-Catechin at 100 μM produced a marked effect; more than 80% of cells were viable upon UVB exposure (P<0.05). These observations indicate that (+)-catechin is effective in the prevention of UVB-induced human keratinocyte damage.

**(+)-Catechin inhibits UVB-induced H2O2 production in keratinocytes**

Previous studies have shown that H2O2 is generated and is responsible for cell damage in cultured human skin cells during UVB irradiation (Peus et al., 1999a; Katiyar et al., 2001). We therefore tested if (+)-catechin can inhibit intracellular H2O2 production. The intracellular H2O2 in keratinocytes was measured by using DHR 123, a dye has been shown to react with H2O2 in the presence of peroxidase and is extensively used as a probe for the detection of intracellular H2O2 (Peus et al., 1999b; Katiyar et al., 2001). Flow cytometric analysis showed that mean fluorescence, i.e. intracellular H2O2 production, was increased about 1.3- to 1.5-fold in UVB-treated cells compared to untreated control cells (Fig. 2a,b). However, the increase of intracellular H2O2 was inhibited by treatment with (+)-catechin in a concentration-dependent manner (Fig. 2c–e). Treatment of cells with (+)-catechin (10 μM and 100 μM) significantly inhibited intracellular H2O2 production (Fig. 2f) (P<0.05 compared with control). The observation indicates that (+)-catechin may have potent scavenging activity, preventing intracellular H2O2 production when keratinocytes are challenged with UVB.

**(+)-Catechin inhibits UVB-induced JNK activation**

UVB irradiation has been shown to cause the activation of ERK, JNK, and p38 kinase (Katiyar et al., 2001; Maziere et al., 2001), which may lead to skin cell damage. In our experimental system, we also observed that ERK1/2, JNK, and p38 phosphorylations were apparently increased in UVB-irradiated keratinocytes (Fig. 3). However, only JNK activation in
keratinocytes was markedly reduced by the treatment with (+)-catechin. UVB-induced ERK1/2 and p38 activation was not affected by (+)-catechin even at higher concentrations (Fig. 3a, b, c, upper panels). Reprobing of the immunoblots with antibodies raised against total JNK, ERK1/2, and p38 demonstrated even loading of each sample (Fig. 3a, b, c, lower panels). Past studies have shown that EGCG can inhibit UVB-induced JNK, ERK, and p38 phosphorylation in keratinocytes (Katiyar et al., 2001). Our result demonstrates that (+)-catechin mainly affects JNK activation and inhibits UVB-induced keratinocyte death.

(+)-Catechin inhibits H$_2$O$_2$-induced JNK activation and cell death

We next examined whether (+)-catechin affects the activation of kinases induced by H$_2$O$_2$. As shown in Fig. 4, H$_2$O$_2$ treatment induced the activation of JNK, ERK1/2, and p38, similar to the effects observed in UVB-irradiated cells (Fig. 4). Again, only JNK activation was markedly inhibited by (+)-catechin (Fig. 4a). (+)-Catechin had little inhibitory effect on H$_2$O$_2$-induced ERK and p38 activation (Fig. 4b,c). As observed in UVB-treated cells, (+)-catechin also reversed H$_2$O$_2$-induced cell death via inhibition of JNK activation (Fig. 5). Treatment of cells with (+)-catechin (10μM and 100μM) significantly increased the cell viability; about 70%–80% cells remained viable (P<0.05 when comparing (+)-catechin-treated cells with H$_2$O$_2$-treated cells not preincubated with (+)-catechin). Taken together, our results indirectly demonstrate that UVB- and H$_2$O$_2$-induced kinase activation occur via similar signaling pathways. Additionally, we demonstrate that (+)-catechin is able to inhibit UVB- and oxidative stress-induced keratinocyte death through a JNK-dependent pathway.

Fig. 2. (+)-Catechin inhibits intracellular H$_2$O$_2$ production in keratinocytes. Keratinocytes preincubated with PBS (a and b) or the indicated concentrations of (+)-catechin (c–e) were loaded with DHR 123. After UVB irradiation (b–e), cells were collected and subjected to flow cytometric analysis. Results are presented as histograms of cell number versus fluorescence intensity. This is representative of four independent experiments. (f) Quantitative analyses of intracellular H$_2$O$_2$ in keratinocytes (n=4). *P<0.05 vs. UVB-exposed cells without (+)-catechin pretreatment.
Fig. 3. Effect of (+)-catechin on UVB-induced JNK, ERK and p38 phosphorylation. Human keratinocytes were preincubated with (+)-catechin for 2h and followed by UVB irradiation (50 mJ/cm²). After a further incubation, cells were collected and lysates were analyzed by Western blot analysis. The result is the representative of five to seven experiments.

Fig. 4. Effect of (+)-catechin on H₂O₂-induced JNK, ERK and p38 phosphorylation. Human keratinocytes were pretreated with (+)-catechin for 2h and followed by the treatment of H₂O₂ (200 μM) for 2h in the absence or presence of (+)-catechin. Cells were collected and lysates were analyzed by Western blot analysis. The result is the representative of five to seven experiments.

Fig. 5. (+)-Catechin prevents H₂O₂-induced cell death. Keratinocytes were pretreated with different concentrations of (+)-catechin for 2h and then challenged with H₂O₂ (200 μM) for 1h in the absence or presence of (+)-catechin. Cell viability was assessed by MTT assay. Results are expressed as percentage of control and are mean±S.E. (n=5). *P<0.05 vs. UVB-exposed cells without (+)-catechin pretreatment.

Fig. 6. (+)-Catechin is less toxic than EGCG on keratinocytes. Keratinocytes were treated with PBS (control) or the indicated concentrations of (a) (+)-catechin or (b) EGCG for 24h. Cell viability was assessed by MTT assay. Results are expressed as percentage of control and are mean±S.E. (n=4). *P<0.01 vs. control.
(+)-Catechin is less cytotoxic than EGCG on keratinocytes

Fig. 6 shows that keratinocytes were viable when incubated with high concentrations of (+)-catechin (up to 400 mM), as determined by MTT and trypan blue exclusion assays (Fig. 6a; data not shown). In contrast, EGCG at 100, 200, and 400 mM significantly decreased the cell viability about 20%, 40% and 60%, respectively (Fig. 6b). This result indicates that (+)-catechin is relatively safer than EGCG.

Discussion

UV irradiation and H2O2 have been reported to upregulate the expression of transcription factors (Devary et al., 1992; Bender et al., 1997), which is mediated by the sequential activation of cytoplastic protein kinases. Peus et al. (1999b, 2000) have demonstrated that ERK, JNK, and p38 are activated in human epidermal keratinocytes following exposure to UVB irradiation. Several lines of evidence have supported that the activation of these kinases correlates with skin cell death in response to UVB (Assefa et al., 2005). However, it has also been suggested that transient activation of ERKs by UV is mainly responsible for skin cell proliferation and differentiation (Cobb and Goldsmith, 1995) and involved in tumor promotion processes (Guyton et al., 1996b). While the evidence indicates that these kinases are activated during keratinocyte apoptosis (Assefa et al., 2005), which of them is mainly responsible for H2O2-induced keratinocyte death remains unclear. Assefa et al. (2000) have shown that UVB irradiation induces keratinocyte apoptosis and produces a sustained p38 activation. However, gene disruption studies indicate that JNK mediates UV-stimulated apoptosis (Lei and Davis, 2003). In this study, we found that (+)-catechin mainly affected JNK activation and inhibited death in keratinocytes upon UVB irradiation (Figs. 1 and 3), implying that JNK is the critical mediator in causing keratinocyte death after UVB exposure. The hypothesis was confirmed by the observations that (+)-catechin also inhibited H2O2-induced JNK activation and cell death (Figs. 4 and 5). These observations are quite different from the effects elicited by EGCG in human cultured keratinocytes, where it inhibits UVB-activated JNK and killed MAPK family members, including ERK1/2, p38 and JNK (Katiyar et al., 2001). Our results indicate that the inhibition of JNK by (+)-catechin is sufficient to reverse UVB- and H2O2-induced cell death. Based on these observations, we suggest that UVB-induced JNK activation is essential in causing keratinocyte death, although some studies have shown that an inhibitor of p38 is capable of inhibiting ~70% of UVB-induced keratinocyte death (Assefa et al., 2000). On the other hand, since (+)-catechin has less inhibitory effect on ERK1/2 activation, suggesting that it possesses relatively weak antiproliferative activity.

Regarding the action mechanisms of (+)-catechin in preventing keratinocyte death upon UVB irradiation, we suggest it acts mainly through its antioxidant effect (Lotito and Fraga, 1998). There is evidence that antioxidants can attenuate MAPK activation (Guyton et al., 1996a; Wilmer et al., 1997; Wang et al., 1998), thereby suggesting that MAPK signaling cascades are important targets affected by ROS levels in cells. Flow cytometric analysis showed that intracellular H2O2 generation was attenuated in the presence of (+)-catechin (Fig. 2). In addition, H2O2-induced JNK activation and cell death were inhibited by (+)-catechin (Figs. 4 and 5), suggesting that (+)-catechin is an antioxidant, which is capable of scavenging of ROS. Shin et al. (2005) have suggested that the accumulation of reactive oxygen species due to catalase attenuation is a critical aspect of the MAP kinase signaling changes that may lead to skin aging and photoaging in human skin in vivo. Jeon et al. (2003) demonstrated that dietary supplementation with (+)-catechin could protect epidermal cells against UVB-induced damage by modulating antioxidant enzyme activities. Therefore, (+)-catechin may reduce the production of H2O2 in keratinocytes through modulating antioxidant enzymes, especially catalase.

In conclusion, the data presented in this study demonstrate that (+)-catechin protects keratinocytes against UVB- and H2O2-induced keratinocyte death. One of the important findings is that (+)-catechin prevents keratinocyte death mainly through the inhibition of UVB- and H2O2-induced JNK activation, which is quite different from the mechanism of action of EGCG. This implies that JNK signaling pathway is critical in UVB- and H2O2-induced keratinocyte death. Moreover, our study demonstrates that (+)-catechin produces less cytotoxicity than EGCG does, indicating (+)-catechin may have large “therapeutic index” when developed as an anti-photoaging agent. Together, we provide here the first evidence that (+)-catechin has novel photoprotective activity. This study not only clarifies the possible death pathways induced by UVB irradiation and oxidative stress but also provides invaluable information for developing effective and cheaper agents for skin care against photoaging.

Acknowledgements

This work was supported by the research grants of the National Science Council of Taiwan (NSC 94-2320-B-030-008) and Shin Kong Wu Ho-Su Memorial Hospital (SKH-FJU-94-15).

References


