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Effect of Cyanidin-3-*O*-glucoside on UVB-Induced Response in Human Keratinocytes

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One of the most significant risk factors associated with the development of skin disease is exposure to UVB radiation from the sun. In particular, UVB light can activate inflammatory and apoptotic pathways, leading to skin damage. Anthocyanins, a group of flavonoids present in many common vegetable foods, are known for their chemopreventive activity. The aim of this study was to evaluate the efficacy of cyanidin-3-*O*-glucoside (C3G) on modulation of cellular responses following exposure to UVB doses in human keratinocytes (HaCaT). In our study, UVB-exposed cells showed significant increase of the translocation of transcription factors NF-kB and AP-1, overexpression of the proinflammatory cytokine IL-8, cleavage of procaspase-3 (a key step in apoptotic pathway), and DNA fragmentation. All these effects elicited by UVB exposure were clearly inhibited by pretreating HaCaT cells with C3G. In conclusion, our data demonstrate that C3G can protect skin cells against the adverse effects of UVB radiation and suggest that it might successfully be employed as a skin photoprotective agent.

KEYWORDS: Cyanidin-3-*O*-glucoside; UVB; skin; inflammation; apoptosis.

INTRODUCTION

UV radiation in the middle-wavelength range (between 290 and 320 nm; UVB) proves to be one of the most relevant environmental risks because of its hazardous effects, which include skin aging (*1*), cancer (*2*, *3*), and immunosuppression (*4*). UVB has a low level of skin penetration, but it can readily affect macromolecules in the epidermal layer, thus altering cellular functions via DNA damage, generation of reactive oxygen species (ROS) (*5*), decrease in skin content of antioxidant compounds (*6*), phosphorylation of receptor-associated tyrosin kinases and protein kinase C (*7*), and activation of the biochemical pathway related to inflammation and apoptosis.

Oxidative stress is thought to play a central role in initiating and driving the signaling events that lead to cellular response and damage following UV irradiation.

Recent studies have suggested that Nuclear Factor-kappaB (NF-kB) and Activator Protein-1 (AP-1) are redox-regulated transcription factors and their activation by different agents, including UV light exposure, is determinant in promoting carcinogenesis (*8*, *9*) by transactivating several classes of target genes related to the inflammatory pathway and immune and cell cycle regulation (*1*, *10*). Among various proinflammatory cytokines, interleukin-8 (IL-8) plays a pivotal role in cutaneous inflammation and has been implicated in tumor promotion and angiogenesis (*10*, *11*). Studies reported that AP-1 and NF-kB-like binding motifs are critical for IL-8 gene induction (*12*).

Sunburn erythema, proposed as one of the earliest examples of apoptosis (*13*), is the most conspicuous and well-recognized acute cutaneous response to UV irradiation, particularly in fair-skinned individuals. The caspase cascade plays a central role in death receptor-mediated signaling pathways that transduce apoptotic signals from the initiator to Caspase-Activated DNase (CAD) via the effector caspases (*14*). Identification of CAD, responsible for internucleosomal DNA fragmentation, and of its proteolytic activation by caspase-3 sheds new light on the molecular basis for the mechanisms of apoptosis (*15*). In particular, UVB induces DNA damage, triggering the apoptosis program via activation of the effector caspase-3 (*16*, *17*).

Primary prevention of skin cancer involves reducing avoidable sun exposure and protecting the skin when sun exposure is unavoidable (*18*). The skin possesses an elaborate antioxidant defense system but, because of constant environmental exposure to physical and chemical agents, an oxidant/antioxidant imbalance results, which greatly influences all stages of skin cancer development. Regular intake of dietary antioxidants or treatment of the skin with products containing antioxidant ingredients may be a useful strategy for the prevention of UV-mediated cutaneous damages.

Anthocyanins represent one of the most important and interesting classes of flavonoids. They are widely spread in dark-

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colored fruits and vegetables such as berries, red grapes, purple sweet potatoes, and red cabbages and seem to play a role in preventing human diseases related to oxidative stress (*19*). These polyphenolic compounds have been reported as having a remarkable spectrum of biological activities; in fact, they possess strong antiinflammatory, antioxidant, antimutagenic, and anticarcinogenic properties and are able to modulate enzymatic pathways (*20*). Cyanidin-3-*O*-glucoside (C3G), largely present in the human diet, is probably the best-known and investigated anthocyanin. Several in vitro and in vivo reports suggest that C3G and derivates possess different biological properties and thus they have potential beneficial effects in various human pathologies (*21*).

There is much evidence supporting that anthocyanins, accumulated in plant skin during acclimation to strong sunlight, can serve as an efficient UVB screen (*22*). In fact, they play an important role in the resistance of the plant photosynthetic apparatus to the UVB component of sun radiation. Furthermore, Tarozzi and co-workers (*23*) have demonstrated the photostability of C3G exposed to UVA−UVB irradiation. However, few data are present in the literature concerning the possible photoprotective activity of C3G in human keratinocyte (HaCaT) cells.

In the present study, we have investigated the in vitro protective effect of C3G on response induced by UVB irradiation in HaCaT cells. We used this cell line because it provides a suitable model to assess the response of epidermal cells to toxic agents. We investigated the effects of C3G on the modulation of NF-kB and AP-1 activation and on the expression of the proinflammatory cytokine IL-8, as a response to UVB irradiation. The effect of C3G activity on UVB-induced caspase-3 activation and DNA fragmentation, considered markers of an apoptosis initiation, was also studied.

MATERIALS AND METHODS

Reagents. Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, L-glutamine, fetal bovine serum, Dulbecco's phosphate buffered saline (DPBS), Trypsin−EDTA solution, trypan blue solution, TRIzol Tris HCl, Triton X-100, DTT, PMSF, Leupeptine, Benzamidine, Aprotine, Bradford reagent, Tween 20, Agarose, SDS, and Ponceau were purchased from Sigma-Aldrich (Milano, Italy).

The C3G was purchased from Extrasynthese (Genay, France) and was HPLC grade.

Anti-caspase-3 antibody and horseradish peroxidase (HRP)-conjugated antibody (IgG) were purchased from BD Biosciences (Milan, Italy)

Dimethyl sulfoxide (DMSO), methanol, ethanol, chloroform, and isopropyl alcohol were obtained from CarloErba Reagent (Milan, Italy) in their highest commercially available purity grade.

Cell Culture and Treatment. The spontaneously transformed human epidermal cell line HaCaT (kindly provided by Dr. N. E. Fusenig, Heidelberg, Germany) (*24*) was cultured in DMEM containing 10% fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin (passage 20−26). The cells were maintained at 37 °C in a humid 5% CO_2 atmosphere incubator.

For all experiments, C3G was always freshly dissolved in DMSO and used immediately. The final concentration of DMSO in the culture medium during different treatments was <0.1% (v/v).

The subconfluent cells were washed with phosphate buffered saline (PBS) under sterile conditions and incubated for 24 h at 37 °C in a humid 5% CO_2 atmosphere incubator at an 80 μM C3G concentration in the culture medium. Control cells were treated with the medium containing only 0.1% DMSO and, after 24 h, were UVB irradiated. Before irradiation, the medium was replaced with a thin film of DPBS.

UVB Irradiation. UVB irradiation was performed using a Philips TL-01 lamp providing radiation in the range of 280−340 nm with an emission peak at 311 nm. Lamp irradiance was 0.2 mJ/cm^2·s as measured by a radiometer (Delta Ohm, Padova, Italy). The system was equipped with an UVC blocking filter. In vitro irradiation of keratinocytes cultures was performed in a six-well plate after replacing the medium with a thin film of DPBS. The cells were exposed to 5, 10, 15, and 20 mJ/cm^2 UVB doses. Sham-irradiated cultures were handled identically, but shielded with aluminum foil during UV exposure. After irradiation, DPBS was removed and replaced with fresh culture medium for 24 h.

Cytotoxicity Assay. The cytotoxic effect of UVB irradiation on HaCaT cells pretreated or not with C3G was evaluated by means of two assay methods, lactate dehydrogenase (LDH) leakage assay (Sigma-Aldrich, Milan, Italy) and trypan blue exclusion assay. The LDH leakage assay evaluates cytotoxicity by measuring activity of lactate dehydrogenase released from damaged cells. LDH activity of both medium supernatant and cell homogenate was measured as the rate of consumption of NADH in the presence of pyruvate. Viability was expressed as the percent of LDH leakage, which corresponds to the ratio between the LDH activity in the medium and the total LDH activity.

To avoid interferences due to antioxidant toxicity, we also used the same tests to evaluate cytotoxicity of C3G pretreatment in HaCaT cells.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total cellular RNA was isolated according to the TRIzol protocol. The quality of the RNA was tested in 1% formaldehyde−agarose gel stained with ethidium bromide (EtBr). The RNA concentration was measured spectrophotometrically at 260 nm using a Beckman DU-60 spectrophotometer (Beckman-Coulter, Milan, Italy). After reverse transcription (RT) with oligo (dT)15 primers, polymerase chain reaction (PCR) was performed for identification of IL-8 mRNA levels. The level of expression of the cytokine was normalized to the expression of the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the same reactions.

Primer sequences: IL-8 forward, 5′ ATG ACT TCC AAG CTG GCC GTG GCT 3′; reverse, 5′ TCT CAG CCC TCT TCA AAA ACT TCT C 3′; GADPH forward, 5′ AGA GAT GGC CAC GGC TGC TT 3′; reverse, 5′ ATT TGC GGT GGA CGA TGG AG 3′.

The following cycling times and temperatures were used to amplify transcripts: 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s for IL-8 and GAPDH. After PCR amplification, the products were visualized by EtBr-stained 1% agarose gel electrophoresis. Band intensities were monitored by densitometric scanning with Scion Image software (Scion Corporation, MD). Moreover, for each gene, increasing amounts of cDNA were amplified in order to check the linearity of the resulting signal. On the bases of these premises, RT-PCR data were considered *semi*quantitative.

Western Blotting. Following appropriate treatment, cell lysates were prepared in nondenaturing lysis buffer (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 μg/mL Leupeptine, 1 mM Benzamidine, 2 μg/mL Aprotinine) and protein concentration was determined using Bradford reagent. For immunoblot analyses, 40 μg of protein lysates per sample were denatured in 2× SDS−PAGE sample buffer and subjected to SDS−PAGE on 12% Tris-glycine gels for Caspase-3.

Separated proteins were transferred to nitrocellulose membrane (Hybond-P PVDF, Amersham Bioscience, Milan, Italy). Residual binding sites on the membrane were blocked by incubation in TBS (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) with 5% (w/v) nonfat milk powder (Amersham Bioscience) for 1 h at room temperature. The membranes were then probed with specific primary antibodies followed by peroxidase-conjugated secondary antibody and visualized with an ECL plus detection system (Amersham Biosciences, Milan, Italy). The equivalent loading of proteins in each well was confirmed by Ponceau staining.

Electrophoretic Mobility Shift Assay (EMSA). The cell pellet was resuspended in 400 μL of ice cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 5% glycerol and protease inhibitors). After a 15 min incubation in ice, cells were lysed by adding 25 μL of 10% Nonidet P40. Nuclei were recovered by centrifugation and lysed in 50 μL ice cold buffer B (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM EDTA, 10% glycerol and protease inhibitors). Sample protein concentrations were brought to 5 μg/μL

with buffer B. Double-stranded oligonucleotides (Life Technologies) were prepared annealing for 10 min 1 nmol of each complementary oligonucleotide in 1× PCR buffer (Promega) by heating at 95 °C for 10 min. Ten picomoles of annealed oligonucleotides were labeled in 1× kinase buffer with 10U T4 polynucleotide kinase (Amersham) and 10 μCi [γ32P]-ATP (NEN) at 37 °C for 40 min. After kinase inactivation, duplex oligonucleotides were purified using Micro Bio-spin30 columns (Biorad). Binding reactions were performed incubating 5 μg of nuclear proteins for 20 min at room temperature with 2 μg of poly dI-dC−Poly-dI-dC (Amersham) and 50000 cpm (Cherenkov counting) of labeled oligonucleotides. DNA protein complexes were resolved in 6% polyacrylamide (29:1 acrylamide/bisacrylamide) gels and then autoradiographed. The specificity of the DNA binding was also confirmed by using binding reactions containing 100-fold excess of unlabeled oligonucleotides as cold competitors (not shown). Oligonucleotide consensus sequences are from 5′ to 3′: NF-kB, AGT TGA GGG GAC TTT CCC AGG C; and AP-1, CGC TTG ATG AGT CAG CCG GAA.

DNA Fragmentation. The pattern of DNA cleavage was analyzed by agarose gel electrophoresis. Briefly, the cell pellets were resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0; 25 mM EDTA; 0.5% SDS, 100 mM NaCl, 200 μg/mL proteinase K) and incubated at 55 °C for 2 h. The cell lysate was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and then centrifuged for 15 min. The supernatant was incubated with RNase A (0.2 μg/mL) at 37°C. After 1 h, the DNA was extracted with phenol and precipitated with one-tenth the volume of 3 M sodium acetate and 3 volumes of 100% ethanol. The DNA samples, dissolved in 1× TE buffer, were separated by horizontal electrophoresis on 1.8% agarose gels, stained with EtBr, and visualized under UV light.

Data Quantification and Presentation. Scion Image software was used for quantification of the transcripts relative abundance in RT-PCR experiments. The figures present one out of at least three independent experiments providing similar results. Data were compared by ANOVA, using a Stat-view package for Apple-Macintosh; differences between groups and treatments were considered significant for $p < 0.05$.

RESULTS

Cytotoxicity of UVB on HaCaT Cells. We evaluated UVB cytotoxicity in HaCaT cells at 3, 7, and 24 h after irradiation. The results show that UVB radiation dramatically affects cellular integrity and viability at doses above 20 mJ/cm^2, as detected by the LDH leakage assay (**Table 1**). The UVB dose of 25 mJ/cm^2 is in fact associated with cellular necrosis already 7 h after irradiation, this effect becoming more evident at 24 h. Trypan blue staining also confirmed low percentage of viable cells after 24 h of UVB exposure (data not shown).

Furthermore, C3G was assessed for its protective effect on cellular damage observed 24 h following UVB exposure (**Table 1**). Pretreatment with a range of concentrations (40−80 μM) of C3G attenuated cell damage in UVB-irradiated cells in a dose-dependent way. In particular, the highest dose of C3G completely inhibited the toxic effect induced by 20 mJ/cm^2 UVB.

Finally, in nonirradiated HaCaT cells, no C3G toxic effect was detected at the concentration of 80 μM utilized in the present study (data not shown).

Since 20 mJ/cm^2 was the lowest UVB dose able to affect cellular integrity and its toxic effect was completely prevented by C3G pretreatment, UVB doses up to 20 mJ/cm^2 were chosen for the successive experiments.

Effect of C3G on NF-kB and AP-1 Activation in UVB-Treated HaCaT Cells. An incubation time from 1 to 9 h after UVB exposure was employed to study the kinetics of AP-1 and NF-kB activation in order to evaluate the optimal time and reproducibility of their activation. **Figure 1** shows the DNA binding activity of NF-kB and AP-1. NF-kB activation increases in cells already at 1 h after UVB dose of 20 mJ/cm^2 and becomes more evident at 7 h. In contrast, in sham-exposed controls, no significant activation was observed. The AP-1 transcription factor is activated 3 h after UVB exposure with an increasing time-dependent trend. Thus, a time of 7 h after exposure to 20 mJ/cm^2 of UVB was chosen to allow an optimal activation of both nuclear factors (**Figure 1**).

Table 1. Survival of HaCaT Cells With (at 24 h) and Without (at 3, 7, and 24 h) Pretreatment with C3G Following UVB Irradiation in HaCaT Cells[a]

Untreated			
	% LDH leakage		
UVB dose (mJ/cm^2)	3 h	7 h	24 h
0	1.0 ± 0.1	2.9 ± 0.2	4.2 ± 3.1
5	1.0 ± 0.4	1.6 ± 0.1	2.8 ± 0.3
10	1.2 ± 0.4	0.5 ± 0.0	1.2 ± 1.9
15	1.2 ± 0.1	1.3 ± 0.1	2.2 ± 1.2
20	1.3 ± 0.1	3.6 ± 0.6	11.7[b] ± 1.8
25	1.5 ± 0.1	8.4[b] ± 1.2	29.2[b] ± 2.5

Pretreated With C3G			
	% LDH leakage		
UVB dose (mJ/cm^2)	control	C3G 40 μM	C3G 80 μM
0	5.0 ± 2.1	2.9 ± 0.2	1.0 ± 0.1
15	1.3 ± 1.2	1.2 ± 0.1	1.2 ± 0.1
20	10.4[b] ± 1.8	5.5[b] ± 0.7	1.4 ± 0.1
25	27.4[b] ± 2.5	15.5[b] ± 0.7	5.5[b] ± 0.7

[a] UVB irradiation was employed at doses ranging from 5 to 25 mJ/cm^2. LDH leakage was determined as the percent ratio between the LDH activity in the medium and the total LDH activity in the cells and medium. Each point represents mean ± SD from three experiments. [b] $P < 0.05$ vs the respective unexposed control.

To evaluate the effects of C3G on the activation of these transcription factors, we analyzed nuclear extracts obtained from C3G-treated and untreated HaCaT cells. **Figure 2A** shows that the activation of NF-kB observed in UVB-exposed control cells was remarkably inhibited by C3G pretreatment.

A similar pattern may be observed in **Figure 2B** for AP-1. In C3G-treated HaCaT cells exposed to UVB, a significant reduction of AP-1 activation was evident in comparison with UVB-exposed control.

Finally, in sham-exposed cells, C3G was unable to activate NF-kB (**Figure 2A**) and AP-1 (**Figure 2B**).

Effect of C3G on UVB-Induced Overexpression of IL-8 mRNA. To establish C3G effects on UVB-induced mRNA expression of IL-8, HaCaT cells were treated with C3G and then irradiated with UVB doses of 10 and 20 mJ/cm^2. IL-8 mRNA expression was assessed, by means of RT-PCR, 24 h after UVB exposure. **Figure 3** shows that IL-8 mRNA expression increases significantly at the UVB dose of 20 mJ/cm^2, while at the UVB dose of 10 mJ/cm^2, no effect on gene expression is detected. Moreover, C3G pretreatment markedly inhibits mRNA expression in UVB-exposed HaCaT cells.

C3G Protects HaCaT Cells from UVB-Induced Apoptosis. To evaluate the activity of C3G on UVB-induced apoptosis, we studied the activation of procaspase-3, a 32 kDa protein cleaved following apoptotic stimuli into a 17 kDa subunit (*16*).

Figure 4 reports the procaspase-3 cleavage for both C3G-treated and untreated HaCaT cells at 10 and 20 mJ/cm^2 of UVB doses. Caspase-3 activation is substantially increased in control

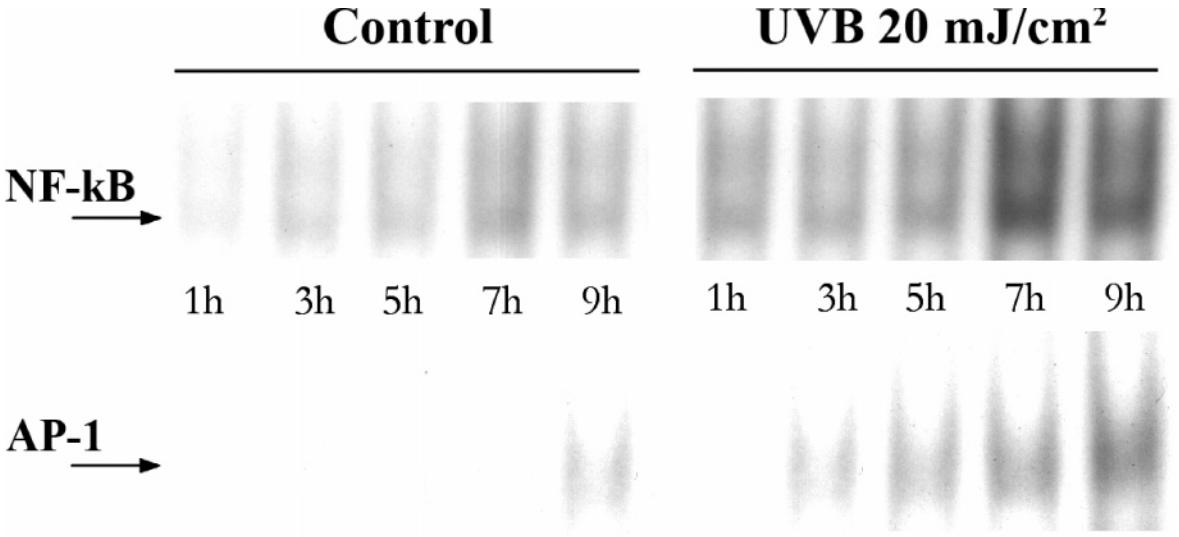

Figure 1. DNA binding activity of NF-kB and AP-1 in HaCaT cells after UVB (20 mJ/cm^2) exposure. Control cells were sham-irradiated. Nuclear extraction was performed from 1 to 9 h after UVB exposure. The specificity of the DNA binding was confirmed by using binding reactions containing 100-fold excess of unlabeled oligonucleotides as cold competitors (not shown).

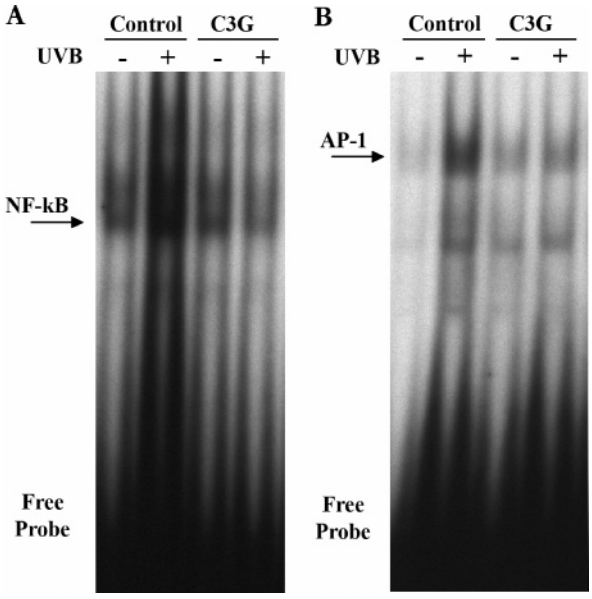

Figure 2. Modulation of NF-kB (A) and AP-1 (B) activation in HaCaT cells by C3G pretreatment, 7 h after UVB exposure. Cells treated with C3G 80 μM or with medium containing only DMSO 0.1% (see Material and Methods) for 24 h, were exposed to UVB (20 mJ/cm^2). Sham-irradiated cells were used as control. Nuclear extraction was performed 7 h after UVB exposure. The specificity of the DNA binding was confirmed by using binding reactions containing 100-fold excess of unlabeled oligonucleotides as cold competitors (not shown).

cells exposed to 20 mJ/cm^2 of UVB. In C3G pretreated cells, UVB-induced cleavage of procaspase-3 is significantly decreased.

As a biochemical hallmark of apoptotic cell death, internucleosomal DNA fragmentation in HaCaT cells was detected. In fact, DNA laddering allows the determination of the amount of DNA degraded upon treatment of cells with certain agents, such as UV. As shown in **Figure 5**, a UVB dose of 20 mJ/cm^2 caused an increase in DNA fragmentation in control cells. However, in C3G-treated cells, UVB did not induce DNA fragmentation, thus supporting C3G protection against UVB damage.

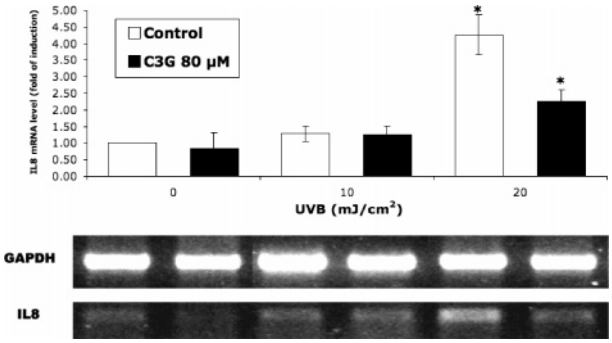

Figure 3. Effect of C3G on the UVB-induced mRNA levels of IL-8 in HaCaT cells. The cells were treated with C3G 80 μM for 24 h followed by UVB (10 and 20 mJ/cm^2) irradiation. RNA extraction was performed 24 h after UVB exposure. The signals of IL-8 mRNA expression were determined by densitometric analysis of the scanned photographs. Relative IL-8 mRNA expression was calculated by normalization to the expression of the internal control GAPDH in each sample, and data are expressed as a fold of induction. Each value represents mean ± SD from three different sets of assays. *, $P < 0.05$ vs the sham-irradiated.

DISCUSSION

Several reports have well demonstrated that supplementation with antioxidants can inhibit skin photocarcinogenesis which, in contrast, may be enhanced by condition increasing ROS generation (*25*). In recent years, a number of studies have suggested that anthocyanins might play a role in countering major acute or chronic human diseases such as cancer (*20*). C3G represents the main and probably the most active compound of this class and its activity has already been demonstrated both in vivo and in vitro (*21*).

To elucidate the capability of C3G to modulate cell response to UVB exposure, we investigated its effects of C3G on signaling pathways involved in inflammation and apoptosis.

NF-kB and AP-1 are key transcription regulators in keratinocytes and play an important role in the regulation of the cell cycle and modulation of different inflammatory pathways. Therefore, particular attention was addressed to the effect of UVB irradiation on the activation of these transcription factors. NF-kB and AP-1 in fact stand out as pivotal factors due to the rapidity of their activation, their unique regulatory mechanisms,

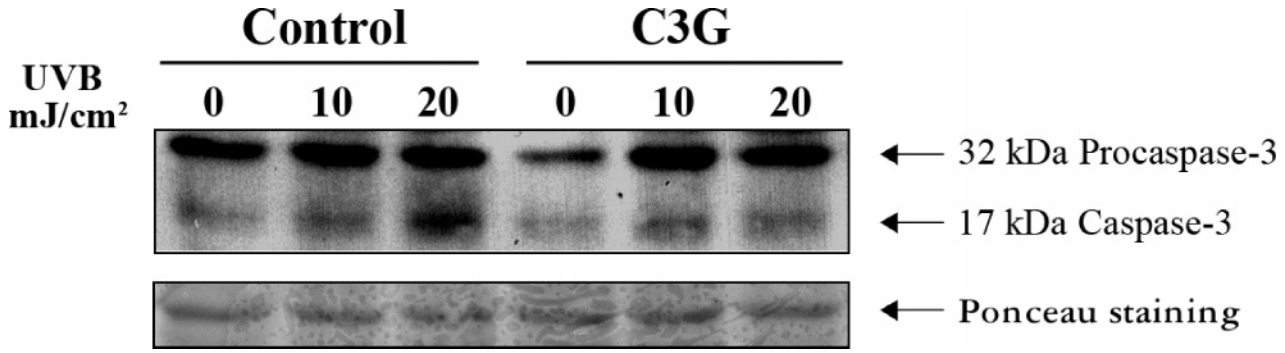

Figure 4. Effect of C3G pretreatment on caspase-3 (active form) activation in HaCaT cells 24 h after UVB exposure. The cells were treated with C3G 80 μM for 24 h and then UVB-irradiated (10 and 20 mJ/cm^2); the cells were lysated 24 h following UVB irradiation.

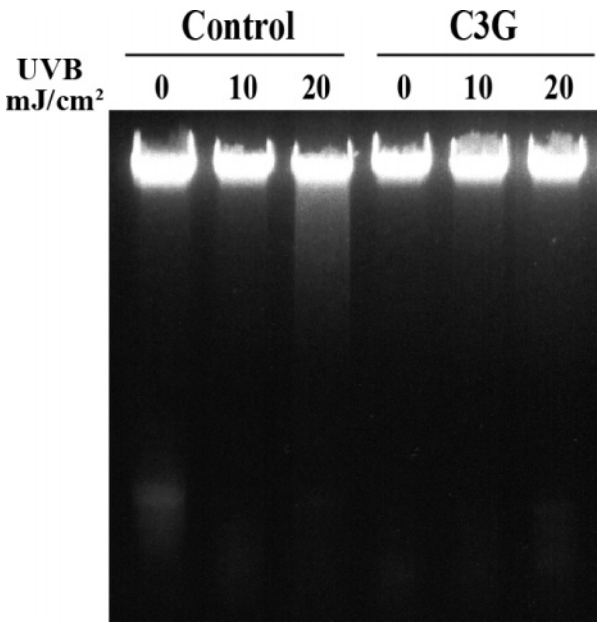

Figure 5. Internucleosomal DNA fragmentation in human HaCaT cells 24 h after UVB exposure. The cells were treated with C3G 80 μM or untreated for 24 h then UVB irradiated (10 and 20 mJ/cm^2), and after 24 h, DNA was extracted and analyzed by agarose gel electrophoresis, followed by ethidium bromide staining.

and the large number of activating signaling pathways and of genes they control. In our study, activation kinetics show an early response of NF-kB following UVB exposure (**Figure 1**). Only a little time in fact is necessary for this transcription factor to translocate into the nucleus after exposure to 20 mJ/cm^2 UVB. A similar temporal expression pattern is shown for AP-1 with a delay of 3 h after UVB irradiation. These findings are in accordance with Saliou and co-workers' observation (*26*) showing an early activation of NF-kB and AP-1 with a similar temporal pattern.

Our study demonstrates that low concentrations of C3G are able to modulate NF-kB and AP-1 activation in HaCaT upon exposure to UVB. In fact, C3G 80 μM totally inhibits translocation of these nuclear factors after 20 mJ/cm^2 of UVB exposure (**Figure 2**). Thus, one can hypothesize that C3G should be able to prevent UVB inflammation in skin. Although many mechanisms may be involved in the up- and down-regulation of IL-8, NF-kB and AP-1 are known to be critical signaling pathways mediating IL-8 expression (*12*). IL-8 cytokine is one of the most important promoters of the initiation phase of cutaneous inflammation (*27*), promoting dendritic cell migration and recruitment of monocytes and neutrophils as key steps in the initiation phase of cutaneous inflammation (*28*). Our results evidence an overexpression of IL-8 mRNA 24 h after 20 mJ/cm^2 of UVB (Figure 3), thus confirming an activation of this inflammatory pathway. C3G pretreatment appears to inhibit IL-8 mRNA expression in UVB-exposed HaCaT cells. Taken together, these findings demonstrate that C3G protects HaCaT cell from UVB-induced inflammation, as observed in transcription factor binding activity and in IL-8 gene expression.

UV interactions with DNA are thought to be major causes in solar skin erythema and sunburn (*2*), suggesting that the main event would be direct damage to DNA by UVB and short UVA wavelengths. For this reason, we evaluated the effect of C3G on UVB induction of the apoptotic pathway. The activation of caspases is required for the accomplishment of the apoptotic program triggered by UVB. In fact, a complete inhibition of UVB-induced apoptosis in HaCaT cells was obtained by treatment with a broad-spectrum caspase inhibitor z-VAD (*29*). The activities of caspase-3, caspase-8, and caspase-9 are all induced according to similar kinetics after UV irradiation. However, caspase-3 can be considered to be subjected to the tightest activation because it is the final, effector caspase at the end of the cascade amplifying the proteolytic activation of all caspases (*30*). In the present study, the UVB dose of 20 mJ/cm^2 seems able to activate caspase-3 cleavage (Figure 4), while at the UVB dose of 10 mJ/cm^2, no activation is observed. C3G inhibits caspase-3 activation following UVB exposure, suggesting that this anthocyanin is able to prevent UVB-induced activation of cell signaling events resulting in apoptosis induction. In agreement with the inhibition of caspase cleavage and activation, the extent of DNA laddering in response to 20 mJ/cm^2 of UVB treatment is markedly decreased following cell pretreatment with C3G.

Our studies indicate that C3G is potentially able to efficiently counter UVB-induced skin damage, very likely by blocking cellular oxidative stress-related events, in particular inflammation and apoptosis. In fact, C3G photoprotection is primarily associated with the down-regulation of NF-kB and AP-1 activation, inhibition of IL-8 overexpression, and prevention of pro-caspase-3 cleavage and DNA fragmentation.

Our findings are in agreement with reports indicating a good in vitro protective effect of anthocyanins against UV-light-induced damage in human keratinocytes (*23*, *31*). One can suggest that the incorporation of C3G by the HaCaT cells reduces the damage elicited by oxidative stress following exposure to UV radiation. Youdim and co-workers (*32*) reported that anthocyanins are incorporated into the membrane and cytosol of endothelial cells and significantly enhance their resistance to the damaging effects of several chemical oxidative stressors. Furthermore Tarozzi and co-workers showed that C3G reduced DNA damage induced by H_2O_2 and $O_2^{\bullet -}$ in HaCaT cells (*23*). Taken together, these observations clearly indicate

that, although exhibiting two major absorption bands in the UV/visible region (250−300 and 450−560 nm ranges), C3G does not only work as sunscreen in our experimental conditions.

Botanical antioxidants also contained in plant-derived food and beverage have a great advantage over existing drugs, as they are nontoxic and inexpensive. Recent studies on the bioavailability of polyphenols confirm their potential therapeutic effects. Among these compounds, C3G has been found to be absorbed unmodified from the diet (*33*) and to have a good bioavailability (*34*, *35*), and some flavonoids have been demonstrated to efficiently penetrate the skin layer (*36*−*38*). The range of concentrations used in the present study is consistent with that employed in many other studies (*23*, *39*−*40*) concerning the protective effect of anthocyanins in culture cells.

Therefore, C3G may be a potential candidate as a complementary photoprotective agent, which could help make sunscreen-based strategies more effective. Considering all these factors, we can propose C3G as a useful natural compound in skin photoprotection with promising applications in the field of dermatology.

ABBREVIATIONS USED

ROS, reactive oxygen species; NF-kB, nuclear factor-kappaB; AP-1, activator protein-1; IL-8, interleukin-8; CAD, caspase-activated DNase; C3G, cyanidin-3-*O*-glucoside; HRP, horseradish peroxidase; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate buffered saline; PBS, phosphate buffered saline; LDH, lactate dehydrogenase; EtBr, ethidium bromide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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