Curcumin from Jianghuang (Rhizoma Curcumae Longae) protects against exposure to ultraviolet B by antioxidation and attenuating mitochondrion-dependent apoptosis

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Abstract
OBJECTIVE: To investigate the protective effect of curcumin extracted from Jianghuang (Rhizoma Curcumae Longae) against ultraviolet B (UVB) and the possible mechanism.

METHODS: Effects of curcumin were detected in vivo and in vitro. Morphological changes of white guinea pig skin were assessed by hematoxylin and eosin staining. HaCaT cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium broide (MTT) assays. The cell cycle distribution, apoptotic rate, level of reactive oxygen species (ROS), mitochondrial membrane potential, and intracellular calcium ion concentration of HaCaT cells were detected by flow cytometry. Antioxidant levels in skin tissues and HaCat cells were measured by biochemical methods.

RESULTS: UVB inhibited in vitro cell proliferation by inducing G2/M arrest, increasing ROS, apoptosis, and necrosis, and decreasing B-cell lymphoma-2, and increasing Bax, cytochrome c, and caspase-3 levels.

CONCLUSION: Curcumin blocks the effects of UVB by reducing ROS and apoptosis, and reversing UVB-induced changes in the expression of apoptotic proteins. The mitochondrial pathway is involved in curcumin-regulated apoptosis.

Keywords: Ultraviolet rays; Curcumin; Antioxidants; Apoptosis; Mitochondria

INTRODUCTION
Jianghuang (Rhizoma Curcumae Longae) belongs to the Zingiberaceae family and grows wild in southeastern Asia and northern Australia with 12 species growing in southern China.13 The essential oil curcumin and its primary and secondary metabolites, which are terpenoids, are principally responsible for the pharmacological activities associated with the plant. Curcumae Longae (C. longa) has recently attracted attention because it exhibits a variety of biological activities including anti-inflammatory, anti-cancer, and neuroprotective effects, and most importantly, a strong antioxidant activity.48 The C. longa essential oil is a natural yellowish orange compound that is soluble in polar organic solvents and...
has been shown to possess anti-inflammatory activities. Studies analyzing the physicochemical and photophysical properties of C. longa essential oil have found that it stimulates angiogenesis. C. longa essential oil consists of several compounds, and curcumin is the most abundant of the curcuminoids (77% of total weight). Curcumin [1,7-bis(4-hydroxy-3-methoxy-phenyl)-1,6-heptadiene-3,5-dione], also called diferuloylmethane, is the primary natural polyphenol found in the rhizome of C. longa (turmeric) and other Curcuma spp. It has been used for centuries as a dietary pigment, spice, and medicine in China. The β-diketone moiety is responsible for the instability and weak pharmacokinetic profile of curcumin. Recent studies have indicated that the β-diketone appears to be a specific substrate for a series of aldo-keto reductases, which is degraded rapidly. The antioxidant activity of curcumin is due to this β-diketone group, and it is responsible for the anti-oxidative and anti-inflammatory properties of curcumin in T lymphocytes. Overwhelming evidence has shown that curcumin has beneficial health effects on cardiovascular, hepatic and neurological functions, cancer, respiratory disorders, and metabolic syndrome, as well as antioxidation and anti-inflammation properties. Ultraviolet (UV) radiation from solar light is a common stressor of human skin. UV radiation is classified according to the wavelength into UVA (320–400 nm), UVB (290–320 nm), and UVC (100–290 nm). UVB represents a small portion of solar radiation, constituting only 5% of all UV rays, but it is the most damaging and genotoxic. UVB directly and indirectly harms skin tissues. After UVB exposure, lipids, proteins, and DNA absorb UVB, generating several pro-mutagenic lesions including cyclobutane pyrimidine dimers and 6-4 photoproducts. Additionally, reactive oxygen species (ROS) are generated excessively, which instigate oxidative stress and lead to cellular toxicity. The resulting oxidative stress is responsible for numerous adverse effects and pathological conditions including cancer. Additionally, UVB exposure reduces keratinocyte viability as evidenced by increased membrane blebbing, cytoskeletal changes, and apoptosis. UVB-induced apoptosis is mediated by apoptotic signals that are divided into two main pathways: intrinsic (mitochondrial) and extrinsic (death receptor dependent).

Recently, interest has grown in the use of natural products that inhibit UVB-induced skin damage because photoprotection with natural products represents a simple and effective strategy to control this problem. Several studies have associated the protective effects of natural extracts against UV-induced skin damage with antioxidant activity and polyphenol and flavonoid contents. Thus, natural antioxidant compounds can inhibit UV-induced ROS and should be considered as an effective barrier against skin damage and photoaging. Based on the antioxidant and anti-apoptotic activities of curcumin, we investigated its protective effect against UVB-induced skin damage and the antioxidant mechanism of curcumin in UVB-exposed guinea pigs. Furthermore, we used N-acetyl-cysteine (NAC) as a positive control drug and explored the molecular mechanism of the antioxidant and anti-apoptotic effects of curcumin in HaCaT cells.

MATERIALS AND METHODS

Animals

White guinea pigs [male, 5 weeks old, (450 ± 50) g] were purchased from the Jilin University Experimental Animal Center (Changchun, China) and routinely fed for 1 week. The animals were then randomly divided into four groups (n = 5 per group): control, UVB, drug (administered before exposure) + UVB, and UVB + drug (administered after exposure).

Establishment of the UVB damage animal model and drug treatments

An area (3 cm × 5 cm) on the back of each guinea pig was cleanly shaved with electric clippers and then exposed to UVB light (Shanghai Rich Light bulb Factory, Shanghai, China; wavelength: 310–315 nm, 15 W × 2). We modified a method by Anbar et al. to meet our experimental needs. The vertical distance was 10 cm from the skin to the light, and the initial dose was 0.168 J/cm². Together with the amount of erythema, doses were increased gradually and the exposure was conducted for 30 d. The total dose was 28.38 J/cm². Curcumin was obtained from School of Pharmaceutical Sciences, Wenzhou Medical University (Wenzhou). Curcumin was dissolved in an oil matrix (3%). When erythema appeared, the skin was smeared with curcumin before or after UVB exposure or with oil matrix after UVB exposure as indicated. The experiment was conducted for more 30 d.

Morphological changes

At the end of the experimental period, the guinea pigs were sacrificed by withdrawing blood under light ether-induced anesthesia, and then samples of dorsal skin were collected. Skin specimens were fixed in 10% formalin and then stained with hematoxylin and eosin (HE) to determine the thickness of the skin epidermis. The tissue sections were dewaxed in xylene, rehydrated through decreasing concentration of ethanol, and washed in PBS, and then stained with hematoxylin and eosin. After staining, sections were dehydrated through increasing concentration of ethanol and xylene, to observe the morphological changes after sealing. The number of fibroblasts in a unit area of dermal tissue was determined under a light microscope (Nikon, Tokyo, Japan) by selecting six fields of view per section from each group and determining average values.
Antioxidant indices and malondialdehyde (MDA) levels
Other skin specimens were used to prepare a 10% tissue homogenate in homogenate medium (0.01 M/L Tris-HCl, 0.001 M/L EDTA-2Na, 0.01 M/L saccharose, and 0.8% NaCl, pH 7.4), according to the manufacturer’s protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Malondialdehyde (MDA) levels, total antioxidant capacity (T-AOC), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) were detected with biochemical kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). For all kits, absorbance values were measured and used to calculate final results by the formulas according to the protocols.

Establishment of the UVB damage model in HaCaT cells
The immortalized human keratinocyte cell line HaCaT was purchased from Peking Union Medical College Bank and incubated in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1% mixture of penicillin and streptomycin (all from Gibco, Grand Island, NY, USA) at 37 °C in a humidified atmosphere containing 5% CO₂. To determine the optimal dose, HaCaT cells were exposed to 10, 20, 30, 40 and 50 μJ/cm² UVB with detection of cell proliferation, the cell cycle distribution and apoptosis.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay
HaCaT cells were seeded in 96-well plates at 6 × 10⁴ cells/well, incubated for 8-12 h, and then exposed to UVB. After 6, 12, 24, 48 and 72 h, 10 μL MTT (5 g/L) was added to each well, and the absorbance value at 490 nm (A₅₇₀) of each well was then read on a microplate reader with gentle shaking. To identify protective effects of treatments, cells were exposed to 30 μJ/cm² UVB followed by 5 mg/L curcumin and 5 nM/L N-acetyl-L-cysteine (NAC). After 18 h, the cells were stained with 10 μL Annexin V-FITC and PI for 15 min in the dark at 4 °C. For ROS, ΔΨm, and [Ca²⁺]i analyses, cells were exposed to 30 μJ/cm² UVB followed by 5 mg/L curcumin and 5 nM/L NAC. After 18 h, the cells were stained with DCFH-DA (20 μM/L), Rh123 (5 μM/L) and Fluo-3 (5 μM/L) for 45 min to detect ROS, ΔΨm or [Ca²⁺]i, respectively. Then, the positive percentages of cells or the mean fluorescence intensity (MFI) were detected by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). For each sample, at least 1 × 10⁴ cells were analyzed. There were four replicate wells per group, and the experiment was performed in triplicate.

Cellular change in antioxidant activity
HaCaT cells were seeded in 6-well plates at 6 × 10⁴ cells/well, incubated for 8-12 h, and then exposed to 30 μJ/cm² UVB followed by 5 mg/L curcumin. After 18 h, the cells were lysed in homogenate medium, and protein concentrations were determined. MDA, NO, NOS, SOD, and GSH-Px were measured according to the protocols provided with the respective kits (Nanjing Jiancheng Bioengineering Institute). There were six replicate wells per group, and all experiments were performed in triplicate.

Western blotting
HaCaT cells were seeded in 6-well plates at 6 × 10⁴ cells/well, incubated for 8-12 h, and then exposed to 30 μJ/cm² UVB followed by 5 mg/L curcumin and 5 nM/L NAC. After 18 h, total protein was extracted and the concentration was measured. Then, 50 μg protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% resolving gel, 5% stacking gel) and transferred to nitrocellulose membranes (200 mA, 1.5 h; Millipore, Billerica, MA, USA). After blocking with 5% nonfat dry milk, the membranes were incubated overnight at 4 °C with diluted solutions (1:200) of the following primary antibodies: anti-β-actin, anti-Bcl-2, anti-Bax, anti-Cyt c, and anti-caspase-3 (Santa Cruz Biotechnology, Dallas, TX, USA). After washing with TBST, the membranes were incubated with IgG-HRP (Santa Cruz Biotechnology, Dallas, TX, USA) as the secondary antibody at a 1:1000 dilution for 2 h at room temperature. Finally, the membranes were developed using a chemiluminescence detection system (ECL detection kit; Santa Cruz Biotechnology, Dallas, TX, USA). The developed films were scanned for subsequent analyses.

Statistical analysis
Statistical analyses were done by the SPSS (statistical package for the social science program, version 24.0, IBM, Armonk, NY, USA) and software for windows. All data are expressed as the mean ± standard deviation. One-way analysis of variance was conducted to
To evaluate the tissue antioxidant capacity, we measured the oxidative stress index (MDA) and several antioxidant indices (T-AOC, SOD, CAT, and GSH-Px). As shown in Figure 1G-K, after UVB exposure, MDA was increased significantly compared with the control, but the antioxidant indices T-AOC, CAT, SOD, and GSH-Px were all decreased significantly (P < 0.05). However, oil matrix had no effect. These results showed that UVB induced oxidative stress in skin. Regardless of curcumin use, the oxidative stress was decreased significantly, while all antioxidant indices were increased significantly (P < 0.05).

**UVR induces changes in HaCaT cell proliferation, the cell cycle distribution, and apoptosis**

The proliferation rates of HaCaT cells exposed to 10, 20, 30, 40, and 50 mJ/cm² were decreased over time (Figure 2A). Additionally, with increasing doses, the percentage of cells in G1/M phase (Figure 2B) and apoptosis rates (Figure 2C-1) were all increased significantly (P < 0.05). UVB originates from solar light, and determine differences between groups with P < 0.05 considered as statistically significant.

**RESULTS**

**Curcumin effects on UVB-exposed guinea pig skin**

The curcumin used in this study was provided by the Biology and Natural Drugs Research Institute of Wenzhou Medical College. Curcumin was dissolved in an oil matrix and used directly. As shown in Figure 1A-E, compared with controls, UVB exposure caused the stratum corneum to disappear and epidermal thickening. Curcumin treatment before or after UVB exposure attenuated this damage, and there were obvious differences between treated skin and control samples, whereas oil matrix had no effect. Additionally, UVB caused vascular changes and increased the number of fibroblasts. Compared with control skin, there were significantly increased numbers of fibroblasts in UVB-exposed samples (P < 0.05; Figure 1F).

Figure 1 Changes in guinea pig skin morphology and oxidative stress indices after UVB exposure are reversed by curcumin A-E: representative images of HE-stained skin sections of Control, UVB, Oil matrix+UVB, Curcumin (3%) + UVB and UVB + Curcumin (3%) groups, changes were found in the stratum corneum (blue arrow), epidermal thickness (black arrow), and fibroblasts (red arrow), ×100; F: changes in fibroblast numbers; G: MDA level changes; H: T-AOC changes; I: superoxide dismutase (SOD) activity changes; J: CAT activity changes; K: GSH-Px activity changes. Data are expressed as the mean ± standard deviation (n = 5). *P < 0.05, compared with the control and **P < 0.05, compared with UVB. HE: hematoxylin and eosin; UVB: ultraviolet B; MDA: Malondialdehyde; T-AOC: total antioxidant capacity; SOD: superoxide dismutase; CAT: catalase; GSH-Px: glutathione peroxidase.
approximately 18-30 mJ/cm² UVB can reach the Earth. Thus, we chose 30 mJ/cm² as the exposure dose for subsequent experiments.

**Curcumin blocks UVB-affected proliferation of HaCaT cells**

We next evaluated the cytotoxicities of various curcumin concentrations and measured their cytoprotective effects after UVB exposure. As shown in Figure 3A and 3B, cells proliferation rates under 1.25, 2.5 and 5 mg/L curcumin treatments were similar to the control, indicating low cytotoxicity. More importantly, the proliferation rate of cells exposed to UVB and 5 mg/L curcumin was increased significantly compared with UVB exposure alone ($P < 0.05$).

**Curcumin reverses UVB-induced oxidative stress**

Based on previous studies, we evaluated the effects of curcumin on UVB-induced oxidative stress. As shown in Figure 4A-I, ROS, NO, NOS, and MDA were high-

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**Figure 2** Proliferation rate, cell cycle distribution, and apoptosis of UVB-exposed HaCaT cells

A: cell proliferation rates after UVB exposure for various times; B: cell cycle distribution after UVB exposure; C: apoptotic percentage changes after UVB exposure: D-I: representative Flow cytometry pictures determined by Annexin V-FITC staining in control, 10, 20, 30, 40 and 50 mJ/cm² UVB groups. The quadrants of the flow cytometry analysis for apoptosis were defined as live (lower left) and apoptosis (lower and upper right). Numbers refer to the percentage of cells in the upper and lower right quadrants. Data are expressed as the mean ± standard deviation ($n = 4$). $P < 0.05$, compared with the control. UVB: ultraviolet B.

**Figure 3** HaCaT cell proliferation rates under various curcumin concentrations with or without UVB exposure

A: cell proliferation rates under the indicated curcumin treatments (1.25, 2.5 and 5 mg/L, respectively); B: cell proliferation rates after the indicated UVB and/or curcumin treatments (1.25, 2.5 and 5 mg/L, respectively). Data are expressed as the mean ± standard deviation ($n = 4$). $P < 0.05$, compared with the control and $^{a}P < 0.05$, compared with UVB. UVB: ultraviolet B.
stress and inflammation. UVB exposure is the major cause of serious skin disorders and genotoxic damage.33,34 Exposing mammalian skin to UV light impairs antioxidant defenses and increases cellular ROS levels, which in turn damage lipids, proteins, and nucleic acids. These processes increase photo-carcinogenesis and photocarcinoma.35,36 Overwhelming evidence has demonstrated the antioxidant and anti-inflammatory properties of curcumin. Our results demonstrated that curcumin alleviated UVB-induced skin damage, decreased oxidative stress, and enhanced the cellular antioxidant capacity.

Using the guinea pig in vivo model, we demonstrated the antioxidant capacity of curcumin. However, to explore the detailed effects and mechanisms of its activity, we performed a series of in vitro experiments in immortalized human keratinocyte HaCaT cells exposed to UVB. There is overwhelming evidence that, along with substantially impeding plant growth, low-dose UVB is an environmental regulator of gene expression, cellular and metabolic activity, growth, and development.37,38 Whether UVB is a stressor or regulator is determined by the fluence rate and exposure time.39 UVB originates from solar light, and approximately 18-30 mJ/cm² UVB can reach the Earth’s surface. Thus,

DISCUSSION

The skin damage induced by UVB involves oxidative stress and inflammation. UVB exposure is the major cause of serious skin disorders and genotoxic damage.33,34 Exposing mammalian skin to UV light impairs antioxidant defenses and increases cellular ROS levels, which in turn damage lipids, proteins, and nucleic acids. These processes increase photo-carcinogenesis and photocarcinoma.35,36 Overwhelming evidence has demonstrated the antioxidant and anti-inflammatory properties of curcumin. Our results demonstrated that curcumin alleviated UVB-induced skin damage, decreased oxidative stress, and enhanced the cellular antioxidant capacity.

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Curcumin is a polyphenol that has been shown to target multiple signaling molecules and demonstrates activity at the cellular level. These diverse activities support its multiple health benefits, and it is available in several forms including capsules, tablets, ointments, energy drinks, soaps, and cosmetics. Despite its well-established safety, some negative side effects have been reported. Some subjects receiving 0.45-3.6 g/d curcumin for 1-4 months reported nausea and diarrhea, and had increased serum alkaline phosphatase and dehydrogenase levels. Our results showed low cytotoxicity of curcumin in HaCaT cells, providing evidence for its safe application. Additionally, curcumin is an antioxidant because of its β-diketone group. Accumulating studies have proposed that curcumin enhances the activities of many antioxidant enzymes, such as CAT, SOD, GSH-Px, and heme oxygenase-1.
and even nitric oxide radicals. In human hepatocyte L02 cells treated with the antimicrobial food additive quinocetone to generate free radicals, curcumin blocks ROS formation by increasing SOD activity and reducing glutathione levels.

To explore the mechanistic details of curcumin activity, we measured apoptosis and the expression of apoptosis-related proteins. Mitochondria are an important organelle for apoptotic regulation. There are two main pathways involved in apoptosis: the extrinsic pathway (death receptor) and intrinsic pathway (mitochondrial dependent). Apoptosis is mainly regulated by caspases and Bcl-2 family proteins. When cells are stressed by drugs or radiation, the mitochondrial membrane permeabilizes. Mitochondria are the source and target of ROS. Thus, apoptosis is triggered by ROS. When mitochondrial integrity is destroyed, the apoptotic pathway is activated and apoptosis inevitably occurs. Apoptosis is regulated by a cascade of reactions, that is, a series of molecules are activated by exogenous stresses; for example, Bcl-2 and Bax as members of the Bcl-2 family. Bcl-2 works primarily by binding to other related proteins that regulate permeabilization of the outer mitochondrial membrane (OMM). Unlike Bcl-2, Bax overexpression promotes cell death. Bax mediates pro-death functions at the OMM where they oligomerize and permeabilize the OMM, resulting in the release of intermembrane space proteins such as cytochrome c, Smac, and endonuclease G. Our results showed that, after UVB exposure, intrinsic death effector pathways were activated, which perturbed the mitochondrial structure and functions, causing decreased mitochondrial ΔΨm, increased [Ca2+], Cyt c release, caspase-3 activation, decreased Bcl-2, and increased Bax, all of which trigger apoptosis. However, curcumin reversed all of these effects of UV exposure, and even achieved the same effects as the antioxidant drug NAC. Thus, we speculate that curcumin attenuates mitochondrion-dependent apoptosis.

In Conclusion, the present study demonstrated that curcumin extracted from C. longa exhibits significant protective effects on guinea pig skin damaged by UVB, which may be due to its antioxidant effect or attenuating apoptosis through the mitochondrial pathway.

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