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Polypeptide from *Chlamys farreri* inhibits UVB-induced HaCaT cells apoptosis via inhibition CD95 pathway and reactive oxygen species

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Abstract

Polypeptide from *Chlamys farreri* (PCF) is a novel marine active product isolated from gonochoric Chinese scallop *Chlamys farreri* which has recently been found to be an effective antioxidant. In this study, we assessed the effect of PCF on UVB-induced intracellular signalling of apoptosis in HaCaT cells. Pre-treatment with PCF significantly inhibited UVB-induced apoptosis in HaCaT cells. PCF strongly reduced the intracellular reactive oxygen species (ROS) level followed by inhibiting the release of cytochrome c. The expression of CD95 and Fas-associating protein with death domain (FADD) was eliminated in a dose-dependent manner by PCF pre-treatment in UVB-irradiated HaCaT cells, followed by inhibition of cleavage of procaspase-8, whose activation induced cell apoptosis. Furthermore, pre-treatment with the ROS scavenger *N*-acetylcysteine (NAC) and the caspase-8 inhibitor z-IETD-fmk was found to effectively prevent UVB-induced apoptosis, suggesting that UVB-induced HaCaT cell apoptosis was partially due to generation of ROS and activation of the caspase-8 pathway. Consequently, the protective effect of PCF against UVB irradiation in HaCaT cells is exerted by suppression of generation of ROS followed by inhibition of cytochrome *c* release and inactivation of Fas-FADD–caspase-8 pathway, resulting in blockage of UVB-induced apoptosis.

Keywords: *Polypeptide from* Chlamys farreri *(PCF), ultraviolet B (UVB), reactive oxygen species (ROS), CD95, apoptosis*

Introduction

Human beings are daily exposed to ultraviolet (UV) radiation from the sun and the skin directly suffers from the deleterious effects of UV radiation. One of the major biological features of ultraviolet radiation (UV) in the middle wave length range (290–320 nm, UVB) is the induction of apoptotic cell death of keratinocytes, which *in vivo* appear as sunburn cells within the epidermis [1]. In fact UVB causes sunburn 1000 times more than UVA and is also more genotoxic [2]. UVB acts mainly in the epidermal basal cell layer of the skin, inducing direct and indirect adverse biological effects, in particular formation of photoproducts, cell cycle growth arrest, photoageing, immunodepression and photocarcinogenesis [3–5]. Dysregulations in UVB-induced apoptosis may have profound impacts on photocarcinogenesis. Therefore, detailed knowledge about the signalling pathways involved in UVB-induced apoptosis is of practical relevance. UVB-induced apoptosis has been recognized as a complex mechanism in which a variety of signalling pathways are involved [6]. Kulms et al. [7] demonstrated that DNA damage, death receptor activation and ROS formation contribute to UVB-induced apoptosis in an essential and independent way.

Rosette and Karin [8] demonstrated that UVB is able to directly activate cell surface receptors by inducing receptor clustering without the need of the

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respective ligand. Accordingly, we and others showed that this applies also for the death receptor CD95/Fas [9,10]. UVB induces clustering of the CD95 receptor and CD95 clustering by UVB is functionally relevant for the induction of apoptosis since inhibition of CD95 clustering by keeping cells at low temperature (4–10°C) during UVB exposure was associated with a partial reduction of UVB-induced apoptosis [9]. Furthermore, transfection of cells with a dominant negative mutant against FADD (Fas-associated protein with death domain), an important member of the CD95 signalling pathway [11,12], resulted in reduction of UVB-induced apoptosis [9], indicating that death receptor activation by UVB does play an important role in UVB-induced apoptosis. Caspase-8 mediates signal transduction downstream of death receptors located on the cell membrane. Activation of the CD95 receptor either by UVB or by an anti-CD95-Ab leads to activation of the upstream caspase-8 subsequently resulting in direct cleavage of the downstream effector caspase-3 as well as of the pro-apoptotic Bid protein, finally resulting in the execution of apoptosis [13,14].

UV is known to be an inducer of reactive oxygen species (ROS), including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O^2$) and hydroxyl radical ($OH^\bullet$) [13,19,22]. These products have been shown themselves to initiate cellular damage and apoptosis [15]. Therefore, ROS have been implicated in cutaneous ageing as well as in the pathogenesis of inflammatory skin diseases and of skin cancer [16–18]. The cytotoxic potential of ROS involves lipid peroxidation which causes changes in the structure of plasma membranes [19] and damaging of the inner mitochondrial membrane resulting in loss of the membrane potential and consequently in cytochrome *c* release into the cytoplasm [20]. Kulms et al. [7] confirmed that ROS generated during UVB irradiation may directly trigger mitochondrial cytochrome *c* release and do not influence death receptor activation, thereby contributing to apoptosis.

Adverse effects of UV irradiation clearly highlight the need for the development and implementation of novel prevention approaches. Therefore, it is suggested that the topical application of radical scavengers and antioxidants like vitamin E, vitamin C and their derivatives may prevent premature skin ageing and indeed these substances are widely used [21]. However, these antioxidants mainly come from terrestrial herbs and plants, we seldom read the reports of polypeptides as antioxidants, especially for those from marine products. Polypeptide from *Chlamys farreri* (PCF, Mr = 879), a new marine polypeptide compound isolated from *Chlamys farreri rejectamenta*, has been recently found to be an effective antioxidant. As an octapeptide, PCF consists of eight amino acids, namely Pro, Asn, Ser, Thr, Arg, Hyl, Cys and Gly. It's a water-soluble polypeptide with purity above 96%. Previous studies of our laboratory indicated PCF could scavenge oxygen free radicals including superoxide anions (O_2^-), hydroxyl radicals (OH·) generated from the reaction systems and effectively inhibit the oxidative activity of $ONOO^-$ [22]. Furthermore, PCF exhibited a direct ROS scavenging activity [23] and protective effects on HeLa cells [24] and hairless mouse skin [25] damaged by ultraviolet A. In this study, we tested whether PCF inhibits UVB-induced HaCaT cell apoptosis via inhibition activation of the CD95 pathway and generation of ROS by UVB.

Materials and methods

Reagents

PCF (purity > 96%) was isolated from *Chlamys farreri*, purified and analysed by HPLC, dissolved in sterile deionized water, and stored at 4°C. Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Co. USA. ROS detection kit, acetyl-*L*-cysteine (NAC), Hoechst 33258 Staining Kit and DNA Ladder was purchased from Beyotime Company (Jiangsu, China). Fas primers and GAPDH were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China); Rabbit polyclonal antibodies against Fas-associating protein with death domain (FADD), caspase-8, cytochrome *c* were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); *β*-actin antibodies were purchased from Beijing Biosynthesis Biotechnology Co. Ltd. (Beijing, China); Horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit) were purchased from Wuhan Boster Biological technology Co. Ltd. (Wuhan, China); Ultraviolet Radiometer were purchased from Beijing Normal University (Beijing, China).

Cell culture and treatment

The spontaneously immortalized human keratinocyte cell line HaCaT, kindly provided by Dr Ding Boxiao (Yonsei University, Korea), was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Co) supplemented with 10% foetal bovine serum (Dalian Biological Reagent Factory, Dalian, China) and penicillin (100 U/ml) and streptomycin (100 mg/ml) and maintained at 37°C with 5% CO_2 in a humidified atmosphere. The cells were randomly divided into five groups including control group, model group, 5.69 mm PCF group, 2.84 mm PCF group and 1.42 mm PCF group. PCF was added to the cell suspension at a final concentration as described above. After incubated for 2 h at 37°C, HaCaT cells were washed twice of PBS. Cells with a very thin layer of PBS were irradiated for 30 min under UVB lamps (Beijing Normal University, China). The wavelength range of

UVB lamps was 290–320 nm with a peak wavelength at 297 nm. The intensity of radiation reaching the cells, measured by an IL700 radiometer (International Light Inc. Beijing Normal University, China), was 11 μw/cm^2. The dosage irradiated to these cells was 20 mJ/cm^2 after 30 min UVB irradiation. Control cells were sham treated as previously described [26]. At 30 min after UVB irradiation, HaCaT cells were harvested for the following experiments.

DNA fragmentation assay

HaCaT cells were dispensed in six well flat-bottomed plates at a density of 1×10^6 cells/well. (1) The cells were pre-incubated with PCF and NAC (Beyotime Biotechnology, Jiangsu, China) for 2 h. Before UVB irradiation, the medium was removed and the cells were washed with PBS (pH 7.2) twice and then PBS was added, followed by UVB irradiation. Then, all the cells were further incubated for 18 h in the medium. (2) The cells were incubated with caspase-8 inhibitor z-IETD-fmk (Enzyme Systems, CA) for 1 h. The cells were then treated as described in (1). Then HaCaT cells (1×10^6 cells) were harvested and washed with PBS twice. The cells were pelleted and suspended in 500 μl cell lysis buffer (150 mm NaCl, 10 mm Tris–HCl (pH 7.5), 10 mm EDTA, 0.5% SDS, 500 mg/l proteinase K) and incubated overnight at 50°C. After incubation, the cell lysate was extracted with phenol/chloroform/isopropyl alcohol (25:24:1, v/v). DNA was precipitated with sodium acetate and ethanol at −20°C overnight and then 4°C centrifuged at 12 000 × *g* for 10 min and then washed with 70% ethanol. DNA was dissolved in TE buffer (10 mm Tris-HC1 (pH8.0), 10 mm EDTA) and was incubated with RNase A (20 μg/l) at 37°C for 30 min. DNA samples were separated by horizontal electrophoresis on 1.5% agarose gels, stained with ethidium bromide and visualized under UV light.

Staining of apoptotic cells with Hoechst 33258

We also conducted Hoechst 33258 staining for the identification of apoptotic nuclei. HaCaT cells were dispensed in six well flat-bottomed plates at a density of 1×10^6 cells/well. The cells were pre-incubated with PCF for 2 h prior to UVB irradiation. After UVB irradiation, the cells were further incubated for 18 h in the medium.Then, cells were washed with PBS and resuspended in the same buffer and stained with Hoechst 33258 staining solution according to the manufacturer's instructions (Apoptosis, Hoechst Staining Kit, Beyotime Biotechnology, China). Stained nuclei were observed under a fluorescence microscope (Leica DBI 4000 B). The number of cells containing apoptotic nuclei among the blue fluorescent protein expressing cells was counted via fluorescence microscopy. In each group, five microscopic fields were selected randomly and more than 700 cells were counted. Apoptotic cell death was then calculated as a percentage of apoptotic cells over the total blue fluorescent protein-positive cells. Three independent experiments were done.

Assay of intracellular reactive oxygen species (ROS)

Intracellular ROS was detected by means of an oxidation-sensitive fluorescent probe (DCFH-DA). The HaCaT cells were pre-incubated with PCF for 2 h prior to UVB irradiation. After UVB irradiation, the cells were further incubated for 30 min. The cells were washed with D-Hank's and incubated with DCFH-DA at 37°C for 20 min according to the manufacturer's instructions (ROS detection kit, Beyotime Biotechnology, China). DCFH-DA was deacetylated intracellularly by non-specific esterase and was further oxidized by ROS to the fluorescent compound 2,7-dichlorofluorescein (DCF). DCF fluorescence (excitation, 488 nm; emission, 520 nm) was then imaged on an inverted fluorescence microscope. The production of intracellular peroxide was also measured using DCFH-DA coupled with spectrofluorometry (Shimadzu RF5301 PC). Fluorescence was quantified with a spectro- fluorophotometer, set at an excitation of 504 nm and an emission of 524 nm.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The expression of human Fas mRNA in HaCaT cells was examined by RT-PCR. Total RNA was extracted according to Beyozol reagent instruction (Beyozol BeyotimeBiotechnology, China). The sequences of specific oligonucleotide primers were as follows: TCTGGACCCTCCTACCTCTGGT and CAGGC CTTCCAAGTTCTGAGTCT for human Fas (Sangon Biological Engineering Technology, Shanghai, China); CGT GGA AGG ACT CAT GAC CA and TCC AGG GGT CTT ACT CCT TG For GAPDH (Sangon Biological Engineering Technology, Shanghai, China). DNA was amplified immediately with a single cycle at 95°C for 3 min and 35 cycles at 94°C for 30 s and 58°C for 30 s and 72°C for 30 s, and a final extension step was taken at 72°C for 7 min. The PCR products were mixed with 2 ml of gel loading buffer, electrophoresed through a 1% agarose gel and visualized by ethidium-bromide staining. The intensity of each band was calibrated to the standard molecular marker on the same gel and then was normalized against the intensity of GAPDH.

Western Blot analysis

HaCaT cells were dispensed in six well flat-bottomed plates. The cells were pre-incubated with PCF for 2 h prior to UVB irradiation. After UVB irradiation, the cells were further incubated for 4 and 6 h. After incubation, all the cells were collected. The cells were

lysed on ice in lysis buffer (20 mm Tris-HC1 (pH 7.5), 150 mm NaC1, 1 mm EDTA, 1 mm EGTA, 1%Triton X-100, 2.5 mm sodium pyrophosphate, 1 mm β-glycerophosphate, 1 mm Na_3VO_4, 1 mg/L leupeptin, 1 mm phenylmethylsulphonyl fluoride). The lysate was centrifuged at 12 000×*g* at 4°C for 5 min. The protein concentration was determined by BCA Protein Assay Kit (Beyotime Biotechnology, Jiangsu, China). Equal amounts of total proteins were mixed in 2×loading buffer (50 mm Tris–HCl (pH 6.8), 2% SDS, 10% 2-mercaptoethanol, 10% glycerol and 0.002% bromphenol blue), boiled for 3 min and subjected to 10% SDS-polyacryamide gel electrophoresis. Proteins were electrotransferred onto nitrocellulose membranes. The membrane was then blocked with 5% BSA in TBST (10 mm Tris–HCl (pH 7.5), 150 mm NaCl, 0.05%Tween20) at room temperature for 1 h and subsequently incubated with rabbit anti-FADD, rabbit anti-procaspase-8, mouse anti-cytochrome *c* (1:200) (Santa Cruz, CA) or anti-β-actin (1:1,000) (Biosynthesis Biotechnology, Beijing, China) antibodies overnight at 4°C. Goat anti-rabbit secondary antibodies or Goat anti-mouse secondary antibody (Boster Biological technology, Wuhan, China) were diluted at 1:2000 in 5% BSA/TBST and were incubated with membranes for 1 h at room temperature. Membranes were washed three times for 5 min each in TBST between antibody incubations. Protein bands were visualized using the ECL Western Blotting kit (Pufei Biotechnology, Shanghai, China). The densities of sample bands were determined using a fluorescence scanner and analysed with Quantity One analysis software (Bio-Rad, USA).

Statistical analysis

Data were expressed as mean values ± SD. The statistical significance was determined by one-way ANOVA. A difference was considered to be statistically significant with $p < 0.05$.

Results

Inhibitory effects of PCF on HaCaT cells apoptosis induced by UVB irradiation

DNA fragmentation, a hallmark of apoptosis, was confirmed using an agarose gel electrophoresis. A typical ladder pattern of inter-nucleosomal DNA fragmentation was observed in UVB model compared with control groups (Figure 1, line 7). DNA fragmentation of all PCF groups (1.42 ~ 5.69 mm) was not obvious, confirming that UVB could directly induce DNA damage and DNA fragmentation was effectively reduced in a dose-dependent by PCF pre-treatment (Figure 1, lines 4–6). The ROS scavenger NAC also demonstrated anti-apoptotic activity (Figure 1, line 3), suggesting that UVB-induced

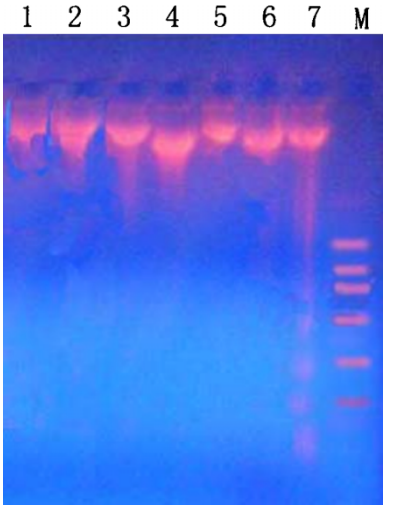

Figure 1. Effect of PCF, NAC, z-DEVD-fmk on UVB induced DNA-fragmentation in HaCaT cells. *M* marker (2000, 1000, 750, 500, 250, 100 bp); *lane 1*: HaCaT cells were mock-irradiated (control), *lane 7*: HaCaT cells were irradiated with 20 mJ/cm^2 UVB, *lane 2*: HaCaT cells were pre-treated with 10 μmol/L z-IETD-fmk for 1 h prior to UVB irradiation, *lanes 3–6*: HaCaT cells were pre-treated with 5 mm NAC or 1.42, 2.84, 5.69 mm PCF for 2 h prior to UVB irradiation, respectively. After irradiation, cells were further incubated for 18 h and DNA-fragmentation was detected by agarose gel electrophoresis. Results were representative of three independent experiments (n=3).

apoptosis in HaCaT cells is mediated by ROS. Hoechst 33258 staining, another hallmark of apoptosis, was also observed in the changes in the cells' nuclei. Normal cells showed homogeneous staining of their nuclei. In contrast, when cells were UVB irradiated, apoptotic cells showed irregular staining of their nuclei as a result of chromatin condensation and nuclear fragmentation; some cells exhibited typical apoptotic bleb phenomenon. As shown in Figure 2, pre-treatment with 1.42 and 5.69 mm PCF for 2 h prior to UVB irradiation prevented UVB-induced apoptosis. These data support the view that PCF inhibits effectively UVB-induced HaCaT cells apoptosis in a dose-dependent manner.

Suppression of the UVB-induced ROS generation in HaCaT cells by PCF

Several reports have suggested that UV radiation can induce change in the intracellular ROS level [27]. In order to elucidate the molecular basis of the anti-apoptotic effect of PCF, we determined whether PCF exerts an inhibitive effect on UVB-induced ROS production. We measured intracellular ROS production using the oxidant-sensitive fluorescent dye DCFH-DA. As shown in Figure 3, intracellular ROS generation was detected 30 min after UVB irradiation and obviously repressed in a dose-dependent manner by the PCF pre-treatment. This result strongly indicates that PCF protects HaCaT cells

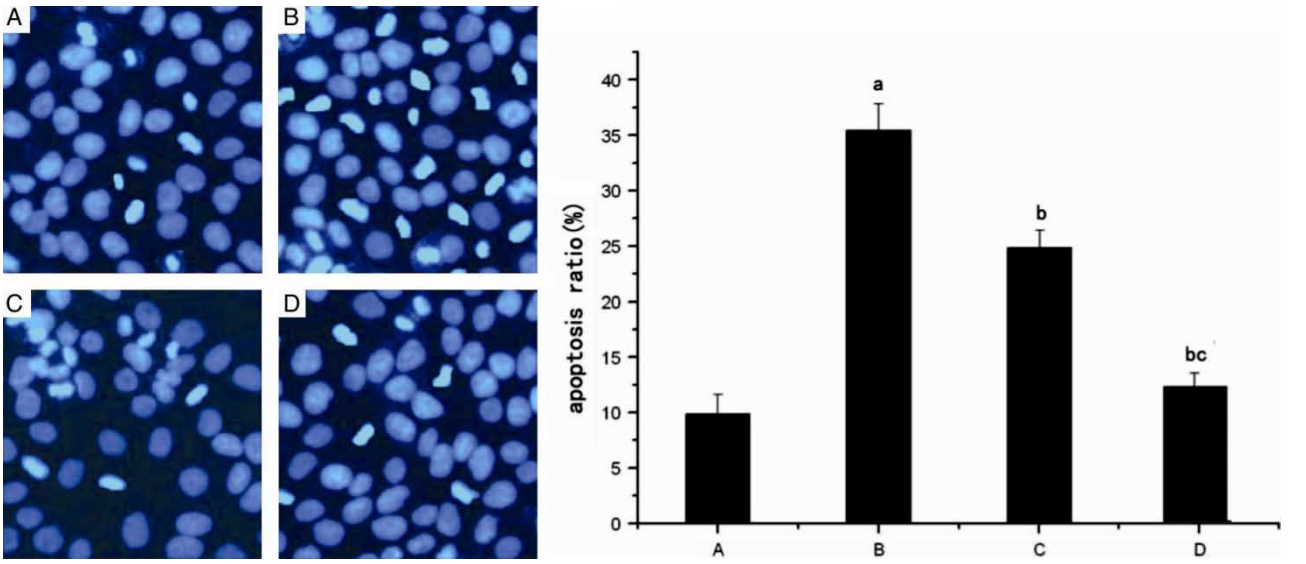

Figure 2. Effect of PCF on UVB-induced apoptosis in HaCaT cells. (A) HaCaT cells were mock-irradiated (control), (B) HaCaT cells were irradiated with 20 mJ/cm^2 UVB, (C and D) HaCaT cells were pre-treated with 1.42 or 5.69 mm PCF for 2 h prior to UVB irradiation, respectively. After irradiation, cells were further incubated for 18 h and apoptotic cells were detected by Hoechst 33258 staining. Indicated are mean ± SD of three independent experiments ($\bar{x} \pm s$, $n = 3$). [a] $p < 0.01$ compared with control, [b] $p < 0.01$ compared with UVB irradiation group, [c] $p < 0.01$ compared with 1.42 mm + UVB group.

from UVB-induced apoptosis via the suppression of intracellular ROS generation.

The release of cytochrome c was reduced in PCF or NAC treated HaCaT cells

Cytochrome *c* is located at the mitochondrial intermembrane space and its release by stimuli such as ROS generation initiates apoptotic cell death by activating the downstream effector caspases (caspase − 3, − 6, − 7). The cytotoxic potential of ROS involves lipid peroxidation which causes changes in the structure of plasma membranes [19] and damaging of the inner mitochondrial membrane resulting in loss of the membrane potential and consequently in cytochrome *c* release into the cytoplasm [20]. Therefore, in this study, we also examined the expression of cytoplasm cytochrome *c* by Western blot analysis. As shown in Figure 4, lanes 3–5, when cells were pre-incubated with PCF for 2 h, the levels of the expression of cytoplasm cytochrome *c* were concentration-dependently decreased. These results suggest that PCF inhibits UVB-induced apoptosis through inhibition of the release of cytochrome *c*. NAC also attenuates release of cytochrome *c*, indicating the possible role of oxidative stress in UVB-induced release of cytochrome *c*.

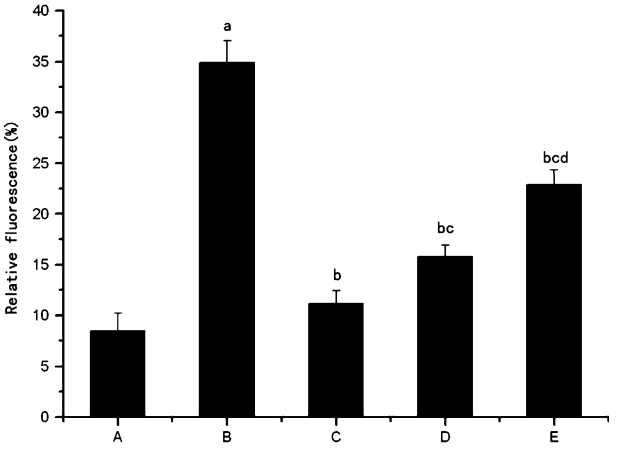

Figure 3. Repression of UVB-induced ROS production by PCF. (A) HaCaT cells were mock-irradiated (control), (B) HaCaT cells were irradiated with 20 mJ/cm^2 UVB, (C, D and E) HaCaT cells were pre-treated with 5.69, 2.84, 1.42 mm PCF for 2 h prior to UVB irradiation, respectively. After irradiation, cells were further incubated for 30 min and the intracellular ROS levels were measured via fluorescence microscopy using the oxidant-sensitive probe DCFH-DA. DCF fluorescence in the cells was quantified with a spectrofluorometer (excitation, 504 nm; emission, 524 nm). The results represent the mean ± SD of values obtained from three separate experiments. ($\bar{x} \pm s$, $n = 3$). [a] $p < 0.01$ compared with control, [b] $p < 0.01$ compared with UVB irradiation group, [c] $p < 0.05$ compared with 5.69 mm + UVB group, [d] $p < 0.05$ compared with 2.84 mm PCF + UVB group

Effect of PCF and NAC on UVB-induced Fas mRNA expression in HaCaT cells

Since activation of CD95 was involved in UV-induced HaCaT cell apoptosis [9], our PCR results showed that UVB irradiation increased the expression of Fas (peaked at 4 h) compared with control groups (Figure 5, line 5). PCF pre-treatment in a dose-dependent manner (1.42 ~ 5.69 mm) caused decreased expression of Fas (Figure 5, lines 2–4), while expression of Fas were not affected by NAC pre-treatment, confirming that PCF inhibited UVB-induced HaCaT cells apoptosis partly through inhibition of the CD95 pathway and UVB-induced

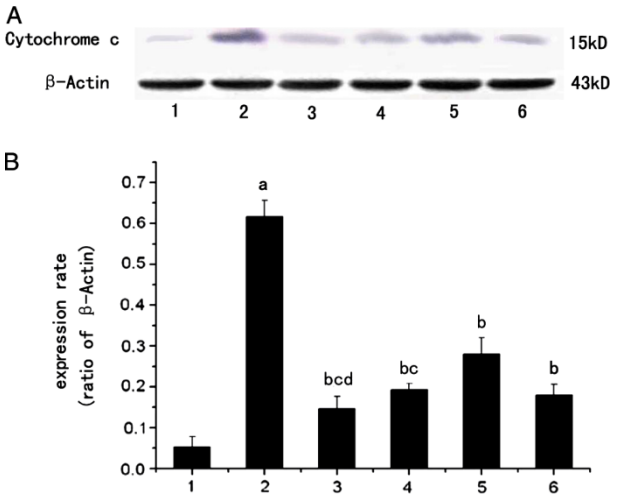

Figure 4. Effect of PCF and NAC on UVB-induced cytoplasm cytochrome *c* protein expression in HaCaT cells. (A) *Lane 1*: HaCaT cells were mock irradiated (control), *lane 2*: HaCaT cells were irradiated with 20 mJ/cm^2 UVB, *lanes 3–6*: HaCaT cells were pre-treated with 5.69, 2.84, 1.42 mm PCF or 5 mm NAC for 2 h prior to UVB irradiation, respectively. Cells were harvested 6 h after irradiation and cytoplasm cytochrome *c* expression were detected by Western Blot. Results shown are representative of three independent experiments ($\bar{x} \pm s$, $n=3$). (B) Quantification of results. Results were expressed as the ratio of expression level of cytochrome *c* over *β*-actin. [a] $p<0.01$ compared with control, [b] $p<0.01$ compared with UVB irradiation group, [c] $p<0.05$ compared with 1.42 mm+UVB group, [d] $p<0.05$ compared with 2.84 mm PCF+UVB group.

ROS generation had no effects on activation of the Fas pathway.

PCF inhibited UVB-induced the expression of FADD in HaCaT cells

UV irradiation could induce HaCaT cell apoptosis via direct activation of CD95, followed by recruitment of FADD to CD95 [9]. Our results showed that UVB irradiation increased the expression of FADD (peaked at 4 h) (Figure 6, line 2). PCF pre-treatment in a dose-dependent manner inhibited the expression of FADD (Figure 6, lines 3–5), which might decrease the recruitment of FADD to CD95, resulting in blockage of the UVB-activated CD95 pathway.

PCF inhibited the cleavage of procaspase-8 in UVB irradiated HaCaT cells

First, to prove that activation of caspase-8 is functionally relevant to UVB-induced apoptosis, the effect of a caspase-8 inhibitor, z-DEVD-fmk, on UVB-induced apoptosis was examined. UVB-induced HaCaT cell apoptosis was reduced when cells were pre-incubated with 10 μmol/L z-DEVD-fmk for 1 h (Figure 1, line 2), suggesting that activation of caspase-8 is not only crucial in CD95-mediated apoptosis, but also indispensable in UVB-induced cell death.

We examined the ability of UVB to activate caspase-8, an initiator caspase that is part of the

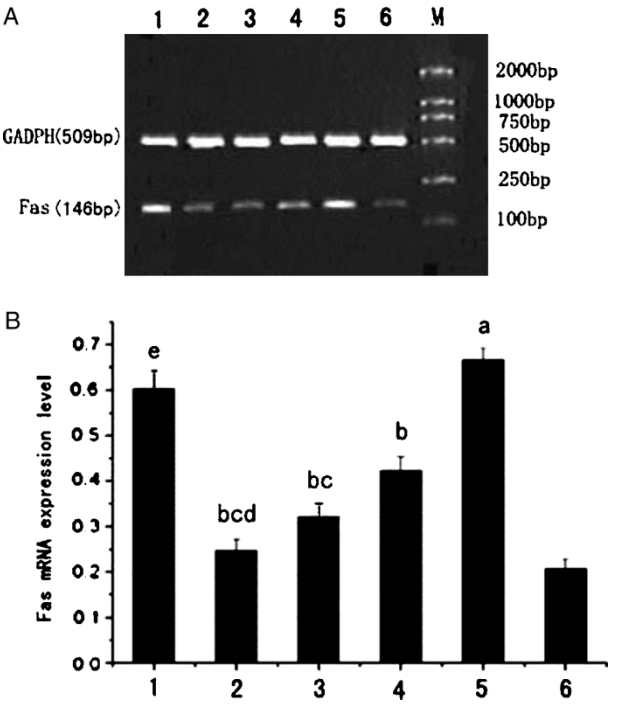

Figure 5. Effect of PCF on UVB-induced Fas mRNA expression in HaCaT cells. (A) *Lane 6*: HaCaT cells were mock irradiated (control), *Lane 5*: HaCaT cells were irradiated with 20 mJ/cm^2 UVB, *Lanes 1–4*: HaCaT cells were pre-treated with 5 mm NAC, 5.69, 2.84 or 1.42 mm PCF for 2 h prior to UVB irradiation, respectively. Cells were harvested 4 h after irradiation and Fas expression were detected by RT-PCR. Results shown are representative of three independent experiments ($\bar{x} \pm s$, $n=3$). (B) Quantification of results. Results were expressed in Fas mRNA normalized against GADPH mRNA. [a] $p<0.01$ compared with control, [b] $p<0.01$ compared with UVB irradiation group, [c] $p<0.05$ compared with 1.42 mm+UVB group, [d] $p<0.05$ compared with 2.84 mm PCF+UVB group, [e] $p>0.05$ compared with UVB irradiation group.

Fas-mediated apoptotic pathway. As shown in Figure 7, in untreated HaCaT cells, an antibody against procaspase-8 detected a protein band of 55 kDa molecular weight, which represents the inactivation of caspase-8. UVB irradiation induced procaspase-8 cleavage into 20 kDa fragments, which became activated and induced HaCaT cells apoptosis. The active fragments of caspase-8 were detectable by Western Blot. PCF pre-treatment in a dose-dependent manner inhibited the cleavage of procaspase-8 (Figure 7, lines 3–5), confirming that PCF inhibited UVB-induced HaCaT cell apoptosis linked to activation of caspase-8.

Discussion

Apoptosis is an evolutionary, highly conserved, biological process requiring the regulated activation of several signalling cascades, which finally result in typical biochemical and morphological alterations of the cell. Many forms of stress can induce apoptosis, including ultraviolet light. Typical

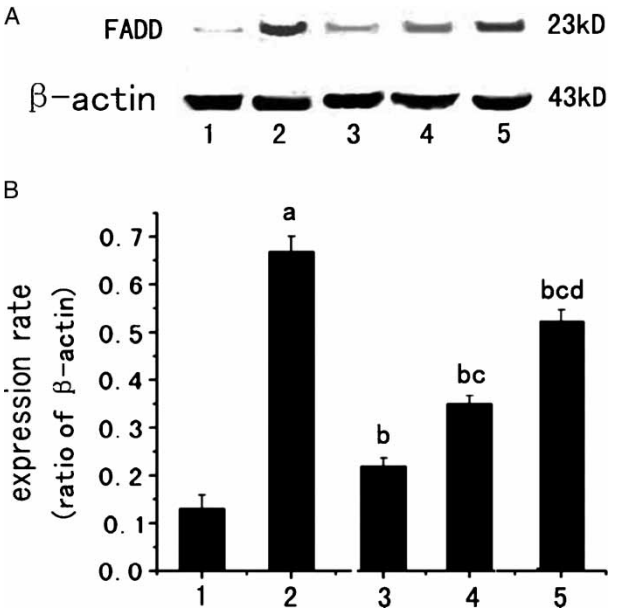

Figure 6. Effect of PCF on UVB-induced FADD protein expression in HaCaT cells. (A) *Lane 1*: HaCaT cells were mock irradiated (control), *lane 2*: HaCaT cells were irradiated with 20 mJ/cm^2 UVB, *lanes 3–5*: HaCaT cells were pre-treated with 5.69, 2.84 or 1.42 mm PCF for 2 h prior to UVB irradiation, respectively. Cells were harvested 4 h after irradiation and FADD expression were detected by Western Blot. Results shown are representative of three independent experiments ($\bar{x} \pm s$, $n=3$). (B) Quantification of results. Results were expressed as the ratio of expression level of FADD over β-actin. [a] $p<0.01$ compared with control, [b] $p<0.01$ compared with UVB irradiation group, [c] $p<0.01$ compared with 5.69 mm PCF+UVB group, [d] $p<0.01$ compared with 2.84 mm PCF+UVB group.

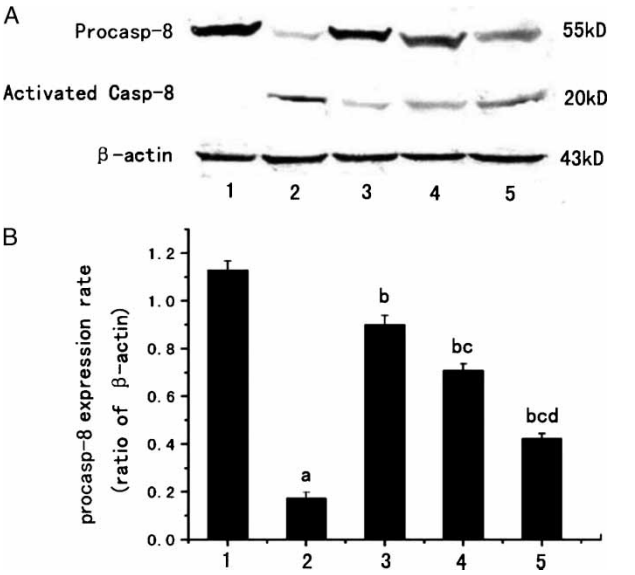

Figure 7. Effect of PCF on UVB-induced procaspase-8 activation in HaCaT cells. (A) *Lane 1*: HaCaT cells were mock irradiated (control), *lane 2*: HaCaT cells were irradiated with 20 mJ/cm^2 UVB, *lanes 3–5*: HaCaT cells were pre-treated with 5.69, 2.84 or 1.42 mm PCF for 2 h prior to UVB irradiation, respectively. Cells were harvested 6 h after irradiation and procaspase-8 activation were detected by Western Blot. Results shown are representative of three independent experiments ($\bar{x} \pm s$, $n=3$). (B) Quantification of results. Results were expressed as the ratio of expressions level of procaspase-8 over β-actin. [a] $p<0.01$ compared with control, [b] $p<0.01$ compared with UVB irradiation group, [c] $p<0.01$ compared with 5.69 mm PCF+UVB group, [d] $p<0.01$ compared with 2.84 mm PCF+UVB group.

changes for apoptosis include condensation of the cell and of the nuclei, DNA fragmentation into nucleosomal units of 200-bp fragments, chromatin condensation, generation of evoluted membrane segments (zeiosis), formation of apoptotic bodies, cellular shrinkage and disintegration of mitochondria [28]. In our study, the results of DNA fragmentation assay and Hoechst 33258 staining manifested that the above phenomenon was significant in model groups, but the phenomenon was dose-dependently reduced by pre-treatment with PCF. This suggested that PCF has the ability to inhibit apoptosis of HaCaT cells induced by UVB.

Kulms et al. [7] indicate that, besides DNA damage and death receptor activation, UVB-mediated ROS formation is the third independent pathway being involved in UVB-induced cell death. Kulms et al. also indicate that CD95 triggering and signalling is independent of ROS and ROS formation during UVB-induced apoptosis cannot only be the consequence of the loss of the mitochondrial membrane potential but may also be directly induced by UVB and cause cytochrome *c* release at early timepoints during UVB-mediated HeLa cell death. Our Western Blot analysis indicates that NAC, the scavenger of ROS, can block cytochrome *c* release and does not influence CD95 activation in UVB-induced HaCaT cells. Lee et al. [29] comfirm that eriodictyol protects keratinocytes from UV-induced apoptosis via the suppression of intracellular ROS generation. It is of great interest, therefore, to see whether or not PCF can protect skin cells against ROS-induced damage. In the present report, our results showed that the ROS level was low in the control group. Upon UVB exposure the ROS level increased and PCF prêt-reatment reduced the ROS accumulation as well as reduction of cytochrome *c* release in HaCaT cells. These results indicate that ROS play an important role in mediating the apoptotic effect of UVB irradiation. UVB-induced HaCaT cells apoptosis can be efficiently attenuated by PCF, suggesting that impairment of ROS formation followed by reduction of cytochrome *c* release during UVB irradiation is responsible for the anti-apoptotic effect of PCF.

Aragane et al. [9] confirmed that UV light activated the CD95 receptor directly by inducing its aggregation and rapidly induced recruitment of FADD to CD95 on HaCaT cells which were of functional importance for UV-induced apoptosis. Zhuang and Kochever [30] concluded that UVA induces rapid apoptosis via activation of the Fas death pathway but independent of FasL in HL-60

cells. These results suggest that the Fas and FADD activation pathway contribute significantly to the UV-induced apoptosis. So it was of interest to examine the protective effect of PCF on UVB-induced HaCaT cells apoptosis through Fas-FADD pathway. Our results showed that when HaCaT cells were irradiated with UVB, the upregulation of Fas and FADD in the model group and the reduction of PCF pre-treatment were clearly detected 4 h after irradiation. These results suggest that PCF inhibited UVB-induced HaCaT cells apoptosis through its effects on Fas-FADD pathway.

In case of the CD95-triggered pathway, caspase-8 is the most upstream located initiator of apoptosis and selectively triggers the activation of different downstream located effectors of apoptosis. In a direct manner caspase-8 proteolytically activates caspase-3 which then cleaves certain death substrates (e.g. lamin, PARP), finally resulting in the execution of apoptosis. Additionally, caspase-8 is known to cleave Bid, which causes promotion of apoptosis [14]. Upon cleavage, bid translocates to the mitochondrial membrane where it interacts with anti-apoptotic bcl-2, leading to mitochondrial megapore formation and finally resulting in cytochrome *c* release from mitochondria which result in the autoproteolytic activation of procaspase-9 [31], whose activation cleaves procaspase-3, a process that culminates in apoptotic cell death. In our experiment, UVB-induced HaCaT cells apoptosis was attenuated by 10 mm z-DEVD-fmk pre-treatment and cleavage of procaspase-8 by UVB irradiation was also reduced by PCF pre-treatment, suggesting that the protective effect of PCF against UVB irradiation occurred through the inactivation of caspase-8.

Taken together, our observations suggest that ROS-cytochrome *c*, Fas-FADD- caspase-8 are involved in UVB-induced apoptosis of HaCaT cells. Furthermore, PCF may affect the ROS/cytochrome *c* and Fas-FADD/caspase-8 signalling pathway in UVB-induced apoptosis. Our previous studies [26] and the present report described the anti-apoptotic and anti-oxidant mechanisms of PCF in HaCaT cells, which could be of great significance in protecting normal human skin against UV-induced cell damage.

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