Inhibitory effect of polypeptides from *Chlamys farreri* on UVB-induced apoptosis and DNA damage in normal human dermal fibroblasts *in vitro*[1]

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ABSTRACT

AIM: To investigate the effect of polypeptide from *Chlamys farreri* (PCF) on ultraviolet B (UVB)-induced apoptosis and DNA damage in cultured normal human dermal fibroblasts. **METHODS:** MTT assay was used to measure the viability of cells. Measurements of apoptosis and cytosolic free $[Ca^{2+}]_i$ were performed with flow cytometry. The comet assay was employed to detect DNA damage in individual cell. **RESULTS:** PCF (0.25 %–1%) greatly enhanced the proliferative capacity of cultured fibroblasts irradiated by UVB (1.176×10^{-4} J·cm^{-2}) and markedly reduced apoptosis and the level of DNA damage in a concentration-dependent manner. Meanwhile, PCF could decrease the cytosolic free $[Ca^{2+}]_i$ ($P<0.01$, compared with UVB model). **CONCLUSION:** The inhibitory effect of PCF on UVB-induced photoaging is due to enhanced abating of UVB-injured DNA and UVB-induced apoptosis. Therefore, PCF can resist UV-induced aging development at the initiation stage.

INTRODUCTION

Human skin is exposed constantly to potentially harmful compounds and radiation. Ultraviolet (UV) radiation is a particularly potent inducer of photoaging. A large number of studies have demonstrated that various kinds of DNA damage accumulate during aging and one of the causes for this could be ambient solar UV radiation. UV-induced apoptotic cell death is considered to play a pivotal role in photoaging.

A number of reports suggest that natural antioxidant can delay skin aging via their capacity of scavenging oxygen free radicals. These reports include studies on vitamin C, vitamin E, SOD, and green tea polyphenols[1]. The bioactivity and the mechanism of their anti-aging are of interest, especially when they prolong life span. If this was the case, it might provide essential clues that ocean may be a source of natural antioxidants.

PCF, M_r=800-1000, is originally isolated from *Chlamys farreri* using bioengineering technique. Previous studies in our laboratory indicated that PCF exhibited direct reactive oxygen species scavenging activity[2] and protective effects on HeLa cells[3] and hairless mice skin[4] damaged by ultraviolet A. As UVB is more harmful to skin than UVA in solar UV radiation, we wonder if PCF could protect skin cells irradiated by UVB. To further elucidate a possible role of PCF in UVB-induced fibroblast cell death, we examined apoptosis in fibroblasts isolated from human dermis and assessed the in-

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fluence of PCF on this process.

MATERIALS AND METHODS

Materials PCF (purity >96 %) was isolated from *Chlamys farreri*, purified and analyzed by HPLC, dissolved in sterile deionized water, and stored at 4 ºC. Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Co. UVB light source was purchased from Beijing Normal University. MTT and Me_2SO were purchased from Sigma Chemical Co. Annexin V-FITC apoptosis detection kit I was obtained from Becton Dickinson Company, Cat No 6693kk. Fluo-3-acetoxymethyl ester (Fluo-3-AM) was purchased from Molecular Probes Inc. Low and normal melting point agarose were supplied by Gibco BRL. Frosted microscope slides were obtained from Curtis Matheson Scientific Inc. 4',6-Diamidine-2-phenylindole dihydrochloride (DAPI) was supplied by Boehringer Mannheim.

Fibroblasts culture A detailed description of human fibroblasts isolation and culture has been published previously[5]. Human dermal fibroblasts were isolated from adolescent foreskin. Briefly, foreskin was treated with 0.25 % dispase for 16 to 18 h. The dermis was separated from the epidermis with forceps. The dermis sheet was then trypsinized for 5 min, and the cells were seeded into 50-cm^2 tissue culture flasks in DMEM supplemented with benzylpenicillin (100 kU/L), streptomycin (100 mg/L) and fetal bovine serum (10 %). The cells were incubated at 37 ºC in a humidified CO_2 (5 %) incubator and split twice per week with alternate 1/3 and 1/4 dilutions. Cells used were passages 4 to 10.

Groups and UVB irradiation Fibroblasts were randomly divided into six groups. They were control group, model group, 0.25 % PCF group, 0.5 % PCF group, and 1 % PCF group. When they were in mid-logarithmic growth phase, the cells were harvested and resuspended at 3×10^7 cells/L, then transferred to 24-well plates triplicate well a group for MTT assay and comet assay with 1 mL as final volume per well. For detection of apoptosis and $[Ca^{2+}]_i$ measurements, cells suspension was seeded into 6-well plates triplicate well a group with 6 mL as final volume per well. After incubation for 48 h at 37 ºC in a humidified CO_2 (5 %) incubator, PCF were added to culture medium at concentrations of 0.25 %, 0.5 %, and 1 %, respectively. After 2-h incubation, cells were exposed to UVB radiation at a dose of 1.176×10^{-4} J·cm^{-2} except those in control group for MTT assay, detections of apoptosis and $[Ca^{2+}]_i$ measurements. The procedure of comet assay was described below.

MTT assay Yellow MTT is converted to the blue formazan product only by metabolically active mitochondria, and the absorbance is directly proportional to the number of viable cells[6]. MTT solution (0.5 g/L) was added to each culture well 6 h after UVB irradiation, and color was allowed to develop for an additional 4 h. An equal volume of Me_2SO was added to stop the reaction and to solubilize the blue crystals. Samples were transferred into 96-well flat bottomed culture plates and the absorbance (A_{490}) was measured at 490 nm using an enzyme-linked immunosorbent assay plate reader[7].

Detection of apoptosis After irradiated by UVB, the cells were incubated for 6 h and then trypsinized. We washed cells twice with cold PBS and resuspended cells in 1×binding buffer at a concentration of 1×10^9 cells/L. We transferred 100 μL of the solution (1×10^5 cells) to a 5 mL culture tube and added 5 μL of Annexin V-FITC and 2 μL propidium iodide (PI). Gently we mixed the cells and incubated for 15 min at room temperature in the dark. Each tube was added 400 μL of 1×binding buffer, then the rates of apoptosis and death were analyzed with Becton Dickinson FACS Vantage flow cytometer as soon as possible (within 1 h)[8]. Histograms were analyzed with FTfit software for each sample, 10 000 events were collected.

$[Ca^{2+}]_i$ measurements Intracellular calcium was determined with the calcium-sensitive fluorochrome Fluo-3[9]. Before loading, cells were incubated for 6 h and harvested. Consequently the cells were washed with cold PBS and resuspended in PBS at a concentration of 1×10^9 cells/L. For loading, cells were mixed with 1:1 with the Fluo-3-AM working solution and incubated for 1 h at 37 ºC in CO_2 (5 %) incubator. During incubation the cells were shaken every 15 min. After 1 h the cells were washed 3 times with cold PBS and analyzed by Becton Dickinson FACS Vantage flow cytometer. Fluorescent signal intensity was examined with Cellguest software. For each sample, 10 000 events were collected.

Comet assay[10,11] Cells grown to confluence in 24-well plates were tripsinized, resuspended in 1mL of medium and irradiated on ice, using a UVB light at a dose of 211.15 J·m^{-2}. Briefly, 80 μL of the cell suspension was mixed with 80 μL of 0.75 % low melting point agarose in PBS, pH 7.4, at 37 ºC giving a final concentration of about 1×10^7 cells/L. Immediately after mixing,

80 μL of the mixture was pipetted on to a frosted glass microscope slide pre-coated with an 80 μL layer of 0.3 % normal melting point agarose. The agarose was allowed to set on ice for 10 min and the slide was immersed in lysis solution (NaCl 2.5 mol/L, edetic acid 100 mmol/L, Tris10 mmol/L, NaOH to pH 10.0, and 1 % Triton X-100) at 4 ºC for 1 h to remove cellular proteins. Slides were then placed in a electrophoresis tank containing NaOH 0.3 mol/L and edetic acid 1 mmol/L, pH >12 for 40 min before electrophoresis at 25 V (1 V/cm, 300 mA) for 30 min at an ambient temperature of 4 ºC. The slides were then washed 3 times for 5 min, each with Tris-HCl 0.4 mol/L, pH 7.5 at 4 ºC before staining with 5 mg/L DAPI. DAPI-stained nucleoids were examined at ×400 magnification with an Olympus fluorescent microscope, equipped with an exitation filter of 350 nm and a barrier filter of 420 nm. One hundred comet on each slide were scored visually as belonging to one of five predefined classes according to tail intensity and given a value of 0, 1, 2, 3, or 4 (from undamaged, 0, to maximally damaged, 4).

Statistical analysis Data were shown as mean±SD. One way ANOVA with Student-Newman-Keuls procedure was used to evaluate difference. *P* level of 0.05 was used to determine significance.

RESULTS

Effect of PCF on UVB-induced cell viability suppression Cell viability was greatly suppressed in the model group. The treatment of PCF (0.25 %-1 %) inhibited the suppression and increased cell viability in a concentration-dependent manner. The viability suppression was completely removed with the addition of PCF 1 %. (Tab 1)

Effect of PCF on UVB-induced apoptosis and cell death The flow cytometric analysis showed that the percentage of apoptosis and death increased in fibroblasts treated with UVB exposure and there was significant difference compared with control group (P<0.05). Treatment of cells with PCF 0.25 %-1 % reduced the rates of apoptosis and death. (Fig 1, Tab 2)

Tab 1. Effect of PCF on UVB-induced cell viability suppression. *n*=9. Mean ± SD. ^{b}P < 0.05 *vs* control. ^{e}P<0.05, ^{f}P < 0.01 *vs* UVB model.

Groups	A_{490}
Control	0.125±0.005
UVB model	0.112±0.004[b]
UVB+0.25 % PCF	0.129±0.004[e]
UVB+0.5 % PCF	0.129±0.007[e]
UVB+1 % PCF	0.135±0.006[f]

Tab 2. Effect of PCF on UVB-induced apoptosis and cell death. *n*=3. Mean±SD. ^{c}P<0.01 *vs* control. ^{e}P<0.05, ^{f}P<0.01 *vs* UVB model.

Groups	Apoptosis rate/%	Death rate/%
Control	1.52±0.04	1.52±0.05
UVB model	5.10±0.09[c]	2.291±0.009[c]
UVB+0.25 % PCF	3.919±0.017[f]	2.074±0.018[e]
UVB+0.5 % PCF	3.56±0.03[f]	1.252±0.024[f]
UVB+1 % PCF	2.237±0.013[f]	1.08±0.04[f]

Effect of PCF on intracellular free calcium level in UVB-treated cells The concentration of intracellular calcium was evaluated by the changes in Fluo-3 fluorescence intensity. PCF 0.25 %-1 % reduced the intracellular free calcium level in UVB-treated cells significantly in a concentration-dependent manner (P<0.01, Tab 3).

Tab 3. Reducing effect of PCF on intracellular free calcium level in UVB-treated cells. *n*=3. Mean±SD. ^{c}P<0.01 *vs* control. ^{f}P<0.01 *vs* UVB model.

Groups	Fluo-3 fluorescence intensity
Control	18.75±0.11
UVB model	31.39±0.12[c]
UVB+0.25 % PCF	28.62±0.11[f]
UVB+0.5 % PCF	24.80±0.10[f]
UVB+1 % PCF	21.62±0.12[f]

Effect of PCF on UVB-induced DNA damage It was found that NHDF exposed to UVB 211.15 $J \cdot m^{-2}$ resulted in serious DNA damage. The damage mainly comprised Grade 4 categories. In contrast, DNA damage was reduced in cells treated with PCF (Tab 4).

DISCUSSION

Recently, we proposed[3] that PCF inhibited UVA-

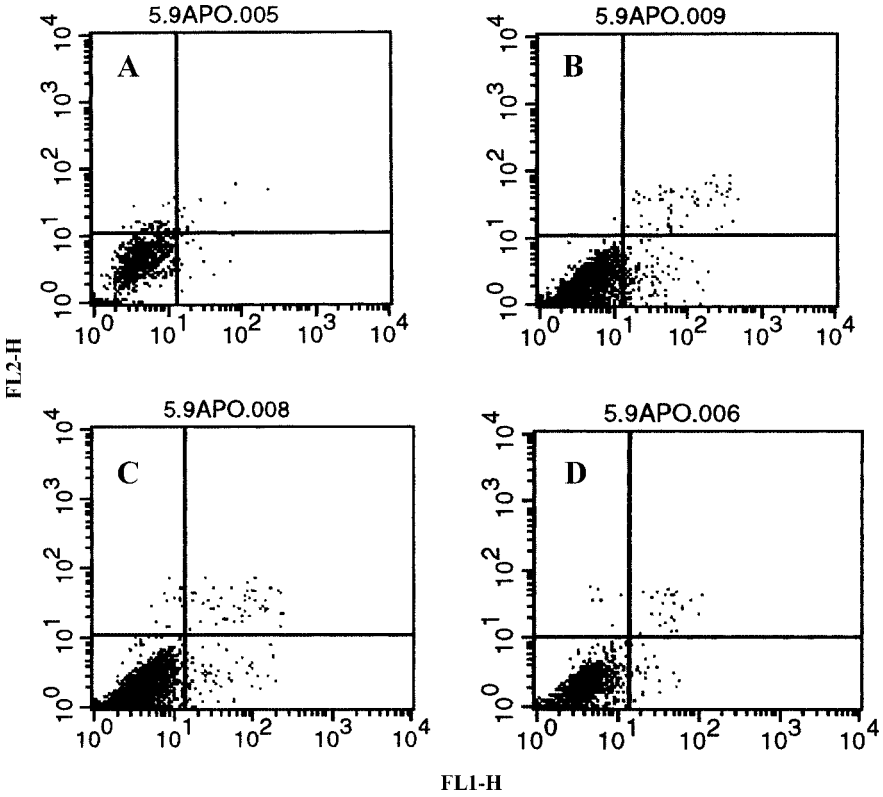

Fig 1. Effect of PCF on UVB-induced apoptosis and cell death (the right upper area shows the death rate and the right down area indicates the apoptosis rate). A: Control; B: UVB model; C: UVB+0.5 % PCF; D: UVB+1 % PCF.

Tab 4. Effect of PCF on UVB-induced DNA damage in NHDF estimated by the comet assay. *n*=3. Mean±SD.

Groups	Grade/%				
	0	1	2	3	4
Control	97±4	1.7±0.3	1.5±0.14	0±0	0±0
UVB model	1.9±0.13	1.3±0.23	2±0.41	9.5±1.3	86±5
UVB+0.25 % PCF	3.6±0.3	2.2±0.5	7.4±0.6	21±2.3	66±7
UVB +0.5 % PCF	5.5±0.4	2±0.7	38±5	34±3	20.7±2.4
UVB + 1 % PCF	6.5±1.09	24±8	56±7	11.7±1.9	2±0.3

induced apoptosis in HeLa cells. To test whether PCF could inhibit apoptosis and DNA damage induced by UVB in fibroblasts we studied the effect of PCF on UVB-induced apoptosis and DNA damage in human dermal fibroblasts.

Our results indicated that PCF possessed a strong protective effect against UVB-induced oxidative DNA damage. UVB irradiation can produce reactive oxygen species and is involved in many physiopathological processes, especially photoaging[12]. In our study, MTT assay was used to measure the viability of fibroblasts. Our results showed that PCF could protect fibroblasts against the insult of UVB.

Strong evidence has shown that reactive oxygen species play a very impotent role in UVB-induced apoptosis[13]. Apoptosis also termed programmed cell death, is a common mode of skin cell death characterized by distinct ultrastructural features and a ladder-like DNA fragmentation pattern[14]. Strong evidence suggests the failure of cells to undergo apoptosis be involved in the pathogenesis of many human diseases, including aging and cancer[15]. Our data showed that

PCF could inhibit UVB-induced apoptosis in fibroblasts. The results suggest that reactive oxygen species scavenging effect may underlie the protective effect of PCF.

Although the antioxidative effect of PCF has been demonstrated in our previous studies, its mechanism has not yet been fully elucidated. In this study, our results showed that PCF-treated cells displayed concentration-dependent reducing effect on UVB-induced Fluo-3 fluorescence intensity, which suggests that PCF suppressed intracellular free calcium accumulation.

UVB is also a well-known genotoxic agent that is able to induce oxidative DNA damage including DNA strand breakage and base modification[16]. In this study, UVB-induced DNA damage was assessed using the comet assay, a simple, sensitive, and reliable method for detecting DNA strand breakage at the individual cell level. Consistent with protective effect on UVB-induced cytotoxicity, PCF displayed a significant protective capability against UVB-induced DNA damage.

In summary, our results demonstrated that UVB induced a decrease in cell viability, an elevation of cytosolic free calcium, and an increase in apoptosis and DNA damage. Although the exact mechanism by which PCF acts remains unknown, our results suggest that the inhibitory effect of PCF on UVB-induced apoptosis and DNA damage are involved in its resistance to UV-induced aging at the initiation stage.

REFERENCES

1 Zhao BL, Li XJ, He RG, Cheng SJ. Scavenging effects of extracts of green tea and natural antioxidants on active radicals. Cell Biophys 1989; 14: 175-84.

2 Wang CB, He MQ, Qin SZ, Wang YZ, Zhong WZ, Xu ZY, *et al*. Antioxidative effect of polypeptide from *Chlamys farreri in vitro*. Chin J Marine Drugs 1998; 3: 15-7.

3 Yao RY, Wang CB. Protective effect of polypeptide from *Chlamys farreri* on HeLa cells damaged by ultraviolet A. Acta Pharmacol Sin 2002; 23: 1018-22.

4 Wang CB, Yao RY, Liu ZT, Zhong WZ, Liu XP, Wang YJ. Protective effects of polypeptide from *Chlamys farreri* on hairless mice damaged by ultraviolet A. Acta Pharmacol Sin 2002; 23: 813-8.

5 Jones GE, Wise CJ. Establishment, maintenance, and cloning of human dermal fibroblasts. In: Pollard JW, Walker JM, editors. Basic cell culture protocols. Totowa: Humana Press; 1997. p 13-21.

6 Mosman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assay. J Immunol Methods 1983; 65: 55-63.

7 Cory AH, Owen TC, Barltrop JA, Cory JG. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Commun 1991; 3: 207-12.

8 Van England M, Ramaekers FC, Schutte B, Rentelingsperger CP. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry 1996; 24: 131-9.

9 Arends MJ. Apoptosis: the role of calcium. Am J Pathol 1990; 136: 593-8.

10 Gredik CM, Ewen SWB, Collins AR. Single-cell gel electrophoresis applied to the analysis of UVC damage and its repair in human cell. Int J Radiat Biol 1991; 62: 313-20.

11 Klaude M, Eriksson S, Nygren J, Ahnstrom G. The comet assay: mechanisms and technical considerations. Mutation Res 1996; 363: 89-96.

12 Epstein JH. Photocarcinogenesis, skin cancer, and aging. J Am Acad Dermatol 1983; 9: 487-91.

13 Dodar DE. Preprogrammed, programmed cell death mechanisms of apoptosis: UV-induce immediate and delayed apoptosis. Photochem Photobiol 1996; 63: 825-36.

14 Majno G, Joris I. Apoptosis, oncosis, and necrosis: an overview of cell death. Am J Pathol 1995; 146: 3-15.

15 Ellis RE, Yuan J, Horvitz HR. Mechanism and functions of cell death. Annu Rev Cell Biol 1991; 7: 663-78.

16 Tornaletti S, Pfeifer GP. UV damage and repair mechanisms in mammalian cells. Bioassay 1996; 18: 221-32.