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Full Length Research Paper

Effects of recombinant human collagen VI from *Escherichia coli* on UVA-irradiated human skin fibroblasts cells

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In this study, we reported the cloning and over expression of a gene coding for human collagen peptide (CP6) in *Escherichia coli* and investigated the protective effects of CP6 on UVA-irradiated human skin fibroblasts cells. The collagen peptide (CP6) was highly soluble and the expression level was approximately 10% of the total bacteria proteins. Also, we performed assays with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Annexin V-fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) methods to investigate the cytoprotective effects of CP6 on the proliferation of UVA-damaged human skin fibroblasts cells. Radiation dosages (5 J/cm²) significantly decreased the proliferation activities of human skin fibroblasts cells (HSF). Compared with UVA irradiated group, in the given concentration, CP6 could improve the activities of cell's proliferation (P<0.05) and decrease the apoptosis rate of cell significantly (P<0.01). UVA could damage the human skin fibroblasts cell *in vitro*. The CP6 had protective effects on HSF irradiated by UVA, and the mechanism of this effect might be associated with its anti-oxidative effect and enhancing cell's proliferation.

Key words: Protein expression, Collagen peptide, Human skin fibroblasts cells, UVA.

INTRODUCTION

UV radiation forms a part of the electromagnetic spectrum with wavelengths between 100 and 400 nm. It is divided into three categories depending on the wavelength, long wave UVA (315-400 nm), medium wave UVB (280 to 315 nm), and short wave UVC (100 to 280 nm) (Svobodová et al., 2003). The UV light that reaches the earth's surface comprises primarily UVA wavelengths and the remainder (approximately 5%) contains the (295-320 nm) UVB radiation (VanderLeun, 2004; Pattison and Davies, 2006). UVA radiation (315-400 nm), which is not

absorbed in the ozone layer, comprises more than 95% of the UV light that reaches the earth. UVA penetrates the epidermis and affects both epidermal and dermal layers of the skin (Lyons and O'Brien, 2002; Mortensen et al., 2008). At a cellular level, UVA-induced damage occurs mainly indirectly via oxidative processes initiated by endogenous photosensitization. UVA exposure causes significant oxidative stress via generation of reactive oxygen species (ROS) such as singlet oxygen, hydroxyl radical, superoxide anion and hydrogen peroxide as well as reactive nitrogen species (RNS), mostly nitric oxide and nitric dioxide (Svobodová et al., 2007). Both UVA and UVB cause wavelength-dependent damage to human skin including skin cancer, whose incidence is dramatically increasing (Pinnel, 2003). UVA penetrates through epidermis deep into the dermis. UVA-induced responses in cells happen mainly because of oxidative processes initiated by endogenous photosensitization. UVA can not only affect numerous signal transduction pathways and impair the immune system but also indirectly produce

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Abbreviations: CP6, Collage peptide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HSF, human skin fibroblasts cells; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

structural damage to the DNA (8-oxoguanine is the most common lesion) and inhibit DNA repair (Svobodova et al., 2006). Apropos the skin photoprotection, to date most of studies have involved UVB protection. Little research has involved prevention against UVA skin damage (Svobodová et al., 2007).

The generation of recombinant proteins is one of the most widely-used techniques in biotechnology research (Makrides, 1996). *E. coli* is one of the most popular hosts for producing recombinant proteins. In addition to its simplicity, safety, and known genetic properties, a major asset is the fact that transformation of *E. coli* with foreign DNA is easy with well-established genetic manipulation methods. So generation of stable cell lines is a speedy process. The major advantage of *E. coli*, however, is its ability to produce proteins in large quantities and to grow very quickly compared with mammalian cells, which enables excellent space/time yields (Swartz, 2001; Baneyx, 1999; Leonhartsberger, 2006). Collagen alpha-2(VI) acts as a cell-binding protein that is involved in the organization of the extracellular matrix (Freise et al., 2009). The collagen extracted from animal bones, hide, and fish scales is called gelatin. The hydrolysate component of collagen is called collagen peptide (CP), and it is used as a dietary supplement. Collagen peptide ingestion may show therapeutic effects on bones, tendons, and skins (Lampe and Bushby, 2005; Merlini et al., 2008; Tanaka et al., 2009). In this study, we described the cloning and expression of the collagen peptide COL6A (or CP6) in *E. coli* and defined a purification protocol. Since the effects of CP6 in the treatment of UVA caused damage have not been reported yet, in this study, we investigated the potential ability of CP6 to ameliorate UVA-induced damage in HSF. The procedure for expression and purification of target protein was produced, which laid a foundation of developing CP6 as a novel drug.

MATERIALS AND METHODS

DMEM, penicillin and streptomycin were purchased from GIBCO-BRL (NY, USA). The restriction enzymes EcoRI and Sall, T4 DNA ligase and Ex Taq were purchased from Sigma Chemical Co. (MO, USA). The DNA gel extraction, plasmid extraction and polymerase chain reaction (PCR) product purification kits were purchased from Takara Co. (DaLian, China). CP6 (purity > 96%) was purified and analyzed by HPLC, dissolved in sterile deionized water, stored at -80°C.

Expression plasmid construction and expression of collagen peptide (CP6)

For constructing a plasmid containing the *Homo sapiens* VI collagen peptide gene, following oligonucleotides were used for PCR amplification: F,R. (F:5'-CCGGAATTCGGCAACAAAGGA GCCAAG-3' and :5'-GCGTTCGACGTTCTTGACAGCCTCCTT-3'). After incubation at 94°C for 5 min, 30 repeated cycles of thermal denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension termination at 72°C for 1 min were performed, followed

by a single step of incubation at 72°C for 7 min. PCR products were analyzed on 1.2% agarose gel, and the desired band was excised from the gel and purified using DNA extraction kit. Then it digested with EcoRI and Sall and ligated into pET32a vector using T4 DNA ligase. The recombinant plasmid containing *H. sapiens* collagen peptide gene was introduced into *Escherichia coli* DE3. DE3 cells were grown in LB medium supplemented with ampicillin, when the OD at 600 nm showed a reading of 0.6-0.7, protein expression was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) (final concentration, 1 mmol/L), and growth was continued for 7 h at 28°C. The cells were collected by centrifugation at 12000 g at 4°C, after the cells were broken by brief pulses of sonication on ice. The supernatant and the precipitate were collected as soluble and insoluble fractions respectively; they were analyzed by SDS-PAGE (Figure 1).

Cell culture

HSF were grown in DMEM supplemented with streptomycin (100 U/mL), penicillin (0.1 mg/mL) and glutamine (4 mmol/L) in a humidified atmosphere with 5% (v/v) CO₂ at 37°C. The cells were subcultured following trypsinization (Krischel et al., 1998). For all experiments, HSF were seeded in plates at a density 1×10⁵ cells/cm² and grown near to confluence.

Cytotoxicity of CP6

Culture medium was removed and cells were treated with serum-free medium containing CP6 (0.1-14 mg/L) at 37°C for 24 h. Control cells were treated with serum-free medium containing DMSO (0.5%, v/v) at the same conditions. Cell viability was evaluated using the MTT assay.

UVA irradiation and treatment

For irradiation, a battery of UVA lamps (Phillips) served as the UVA source. The irradiation intensity was measured using a UVA radiometer (Solar Light, China). For all experiments, HSF cells were irradiated with the UVA dose of 5 J/cm². When grown to 80 to 90% confluence, cells were subjected to irradiation with both UVA lamps (320-400 nm) (PHILIPS) at a dose of 5 J/cm² (Merwald et al., 2005), which were monitored by UVA radiometers (Solar Light, China) respectively.

Cells undergone different treatments: (A) When cells were cultured to 80% confluence, cells were incubated with various doses of CP6 for 24 h. (B) When cells were cultured to 80% confluence, various doses of CP6 (2, 4, 6, 8, 10 mg/mL) were added into the culture, and then cells were exposed to UVA (5 mJ/cm²). (C) When cells were cultured to 80% confluence, cells were irradiated with UVA (5 mJ/cm²). And then the cells were cultured with various doses of CP6 (2, 4, 6, 8, 10 mg/mL) for 12 h. During irradiation, medium was discarded and cells were covered with 2 mL of PBS. At last, we selected the surface of the sun reach the earth close to UVA (5 J/cm²) as the source of irradiation.

MTT assay

Tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), is reduced by intracellular dehydrogenases of viable living cells that leads to formation of purple formazan crystals, insoluble in aqueous solutions. After dissolution in organic solvent, the absorbance is monitored. Serum-free medium supplemented with MTT (5 mg/mL) was applied to the cells for 2 h

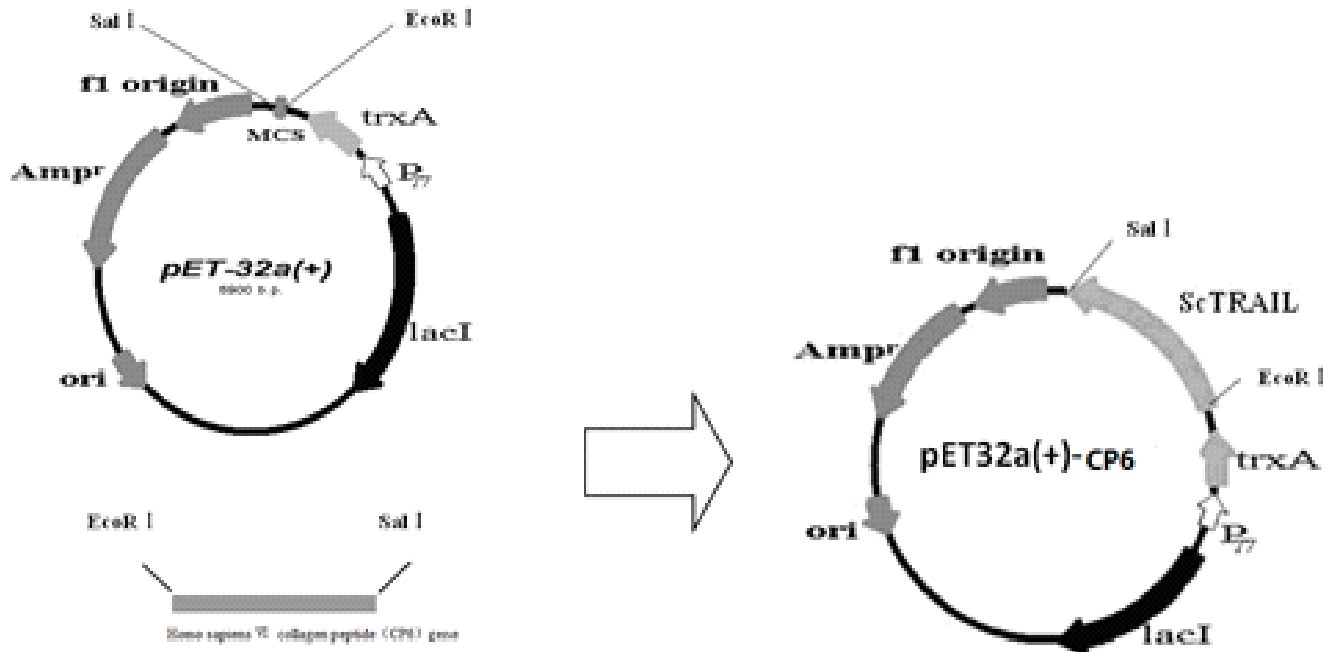


Figure 1. Schematic diagrams of pET-32a (+) expression vector. The recombinant gene encoding Homo sapiens VI collagen peptide was cloned into the pET-32a (+) vector and expressed in *E. coli* BL21 (DE3) strain under the control of T7 promoter.

(37°C). The medium was removed and the crystals were dissolved in DMSO. The absorbance was measured at 570 nm (Pepper et al., 2005).

Flow cytometric analyses of apoptotic and necrotic cells

The treated or control cells were harvested by trypsinization and collected by centrifugation at 300×g for 5 min at room temperature. Cells were washed with cold PBS and stained with Annexin V/PI detection kit (Abcam) according to the manufacturer's instructions. After removing most of the PBS, the cell pellet was resuspended in 400 µL of 1× binding buffer with 5 µL of Annexin V-FITC mixture and gently vortexed. Propidium iodide (10 µL) was then added, and the cells were incubated for 5 min. Data were analyzed using the WinMDI software package.

TUNEL assay

DNA-fragmentation in HSF cells was evaluated by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Shen et al., 2001; Itoh and Horio, 2001). After UVA-irradiation and then incubation, the apoptosis of cell samples was measured by TUNEL staining according to the manufacturer's protocol of TUNEL assay (Roche Diagnostics, Shanghai, China). Briefly, tissue sections on slides were dewaxed and rehydrated by standard protocols (example, by heating at 60°C followed by washing in xylene and rehydration through a graded series of ethanol and double distilled water) (Gavrieli et al., 1992; Portera-Cailliau et al., 1994; Cortés-Gutiérrez et al., 2007), and incubated for 15 to 30 min at 21 to 37°C with Proteinase K working solution. Following two rinses with PBS, 50 L of the TUNEL reaction mixture was added and samples were incubated for 60 min at 37°C in a humidified atmosphere in the dark. Slides were then rinsed three times with PBS and analyzed in a drop of PBS under a

fluorescence microscope. An excitation wavelength in the range of 450 to 500 nm and detection in the range of 515 to 565 nm was used.

Statistical analysis

Results were expressed as the mean ± S.D. All statistical analyses were performed using SPSS v16.0 statistical analysis software. A value of $p < 0.05$ was considered to indicate statistical significance.

RESULTS

Expression plasmid construction and expression of CP6

The nucleotide sequence of Homo sapiens COL6A2, collagen, type VI, alpha 2 (684bp) was determined and submitted to GenBank under accession CR536573.1. In order to construct expression plasmids, PCR was performed with genomic full-length human COL6A2 collagen, typeVI, alpha2 [Homo sapiens] purchased from Origene technologies (USA) as a template and primers. The forward primer contained an EcoRI restriction site, while the reverse primer contained a SalI site. The amplified fragment was digested with EcoRI and SalI and inserted into the EcoRI and SalI sites of pET expression vector, followed by ligation. The plasmids were transferred into *E. coli* DH5a and were then introduced into *E. coli* BL21 (DE3) and expressed under the control of T7 promoter. The expressed collagen peptide (CP6) was about 46 kDa

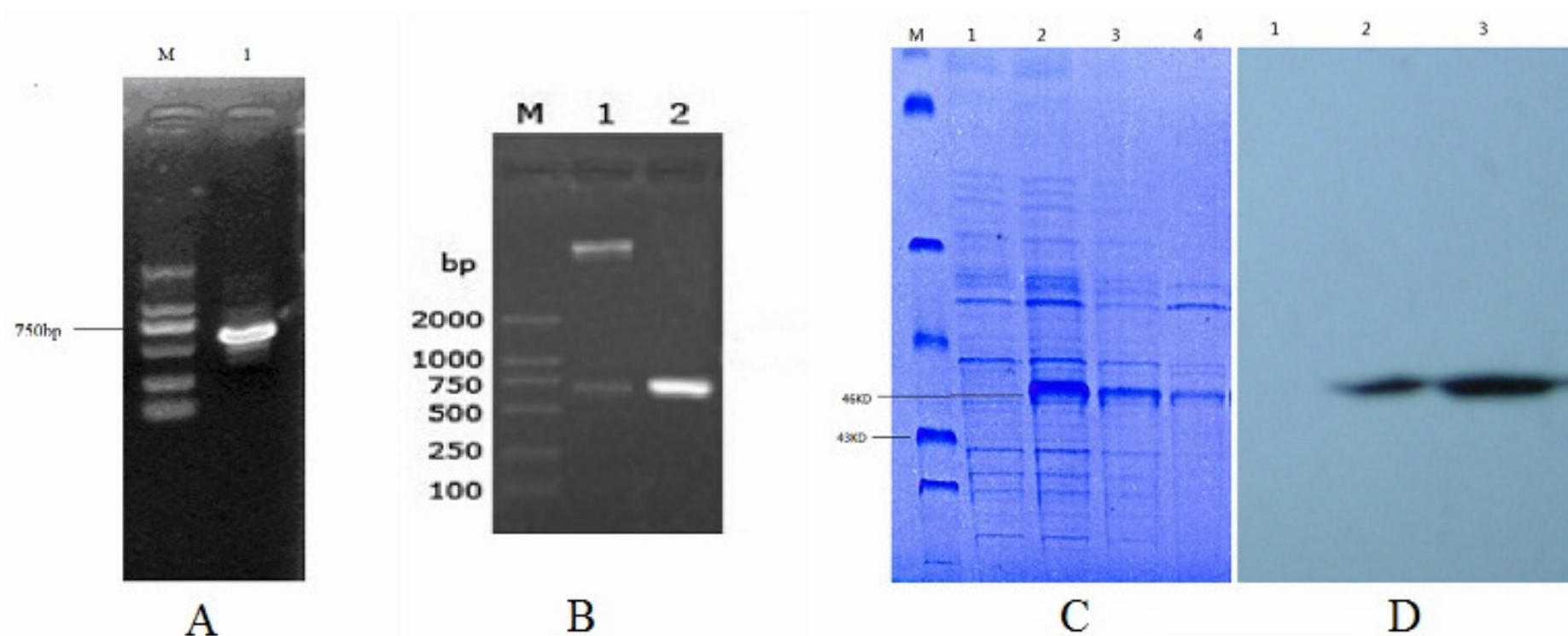


Figure 2. Expression plasmid construction and expression of CP6. (a) The PCR products of target genes. M, DNA marker; lane 1, the PCR fragment of CP6. (b) Restriction analysis of recombinant plasmid pET-32a-CP6. Lane M, DL2000 DNA marker; lane 1, plasmid pET-32a-CP6 digested with EcoRI and Sall. Lane 2, the PCR fragment of CP6. (c) Expression of CP6 in *E. coli* BL21(DE3). Lane M: protein markers; Lane 1: un-induced *E. coli* cell lysate; Lane 2: *E. coli* lysate after IPTG induction; Lane 3: supernatant of the *E. coli* lysate after ultrasonication of the induced *E. coli*; Lane 4: pellet fraction of the *E. coli* lysate after ultrasonication of the induced *E. coli*. (d) Western blotting analysis of the *E. coli* lysate with commercial mouse anti-human collagen VI A2 antibody. Lane 1: un-induced *E. coli* cell lysate; Lane 2: pellet of the *E. coli* lysate after ultrasonication of the induced *E. coli*; Lane 3: supernatant of the *E. coli* lysate after ultrasonication of the induced *E. coli*.

and the expression level was approximately 10% of the total bacteria proteins. SDS-PAGE (Figure 2) analysis revealed that most target proteins existed in soluble fractions. The majority of the expressed recombinant proteins were found to be soluble in the supernatant. Few proteins were found in the soluble fraction. This indicated that the target proteins (CP6) were mainly expressed in the soluble form *E. coli*.

Effects of CP6 on cell viability in UVA-irradiated cells

The effects of CP6 at various doses on HSF cells viability were tested with MTT assay. The results showed that CP6 ranging from 2 to 12 mg/mL had no significant effects on cell viability (data not shown). None of the concentrations tested showed toxicity for the cells. Therefore, we chose

2 and 10 mg/mL for all the experiments.

The effects of CP6 on the viability of UV-irradiated skin cells were assessed. HSF cells were pretreated with the The effects of CP6 on the viability of UV-irradiated skin cells were assessed. HSF cells were pretreated with the CP6 at different final concentrations (2, 4, 6, 8, 10 mg/mL) for 2 h respectively, and then were irradiated with UVA. Cell viability was evaluated

Table 1. Effect of CP6 on UVA-induced HSF cells with the MTT (N=6,*P < 0.01).

Parameter	CP6 added before UVA-irradiation	CP6 added after UVA-irradiation
A Untreated control HSF cells	0.81267 ± 0.009352	0.8189 ± 0.00922
B UVA-treated cells without CP6	0.4385 ± 0.032017	0.4125 ± 0.012017
C UVA+2 mg/mL CP6	0.5065 ± 0.004324	0.4323 ± 0.006364
D UVA+4 mg/mL CP6	0.5545 ± 0.013248*	0.453 ± 0.003288
E UVA+6 mg/mL CP6	0.57333 ± 0.013186*	0.502 ± 0.015256*
F UVA+8 mg/mL CP6	0.65483 ± 0.019874*	0.511 ± 0.02084*
G UVA+10 mg/mL CP6	0.70617 ± 0.006306*	0.515 ± 0.014306*

by the MTT colorimetric assay. As shown in Table 1, UVA-irradiation reduced HSF cell viabilities to approximately 53% of the control values. The survival rate of the cell pretreated with CP6 increased in a dose-dependent manner, and reached maximum value at the concentration of 10 mg/mL.

HSF cells were post-treated with the CP6 at different final concentrations (2, 4, 6, 8, 10 mg/mL) for 8 h respectively. Cell viability was evaluated by the MTT colorimetric assay. As shown in Table 1, the survival rates of the cell post-treated with CP6 increased in a dose-dependent and time-dependent manner.

Moreover, CP6 displayed significantly higher protection effects than therapeutic effects at their maximum efficient concentration of 10 mg/mL. In other words, the CP6 has better preventive effects than its therapeutic effects, so we will perform assays with Annexin V-FITC/PI and TUNEL to investigate the cytoprotective effects of CP6 on the proliferation of UVA-damaged human skin fibroblasts cells.

Effects of CP6 on UVA-induced apoptosis

By the AnnexinV-PI method, cells were both Annexin V- and PI- indicating viability. Since dead cells are PI+, those Annexin V+ but PI- cells are apoptotic. As shown in Figure 3, UVA-irradiation induced a distinct apoptosis rate, which represents the population of apoptotic cells. Statistical analysis showed the apoptotic and dead proportions of the cell pretreated with CP6 decrease in a dose-dependent manner. CP6 administration (10 mg/mL) before UVA-irradiation could significantly decrease the apoptotic and dead proportion of HSF cells by 5.67%. CP6 itself did not cause DNA damage at used concentrations (data not shown). In UVA-exposed cells treated with CP6 a powerful ability to attenuate apoptosis appearance was evident (Figure 3). In UVA-irradiated cells (5 J/cm²) treated with CP6 before UVA-irradiation, an evident diminution of cell apoptosis was observed at all concentrations tested. The reduction was more apparent at a concentration of 10 mg/mL. CP6 was more potent in alleviation of UV-induced apoptosis at used concentrations. CP6 protection was dose-dependent and

was the most efficient at the highest concentration tested.

Effects of CP6 on the DNA-fragmentation in HSF cells

The TUNEL method is a simple and sensitive method for the detection of apoptotic cells. Cells in early stages of apoptosis up to the stage when the morphological changes occur that are typical for apoptosis, including apoptotic bodies, are stained. Every slice selected needed five horizons, each horizon counting 500 cells, and stained positive cells were counted (%). Then the average was calculated. The ImageJ image analysis software processing, the results showed the apoptosis rate (%): A (Untreated control HSF cells) 6.28 ± 3.9352, B (UVA-treated cells without CP6) 40.21 ± 3.4043, C (UVA + 2 mg/mL CP6) 25.89 ± 6.8796, D (UVA + 4 mg/mL CP6) 17.98 ± 4.2435*, E (UVA+6 mg/mL CP6) 10.23 ± 1.4376*, F (UVA + 8 mg/mL CP6) 8.47 ± 1.6548*, G (UVA + 8 mg/mL CP6) 7.98 ± 5.5488* (*P<0.05, n=6). As shown in Figure 4, CP6 administration (10 mg/mL) before UVA-irradiation led to fewer breaking of DNA than that of UVA model group as indicated by the significantly decreased number of labelled cells. The results suggested that CP6 can effectively suppress the apoptosis induced by UVA-irradiation.

DISCUSSION

Human skin is constantly exposed to the UV irradiation in sunlight. This can induce a number of pathobiological cellular changes. The development of novel preventive and therapeutic strategies depends on the molecular mechanisms of UV toxicity. A recent study indicates that UVA may play a pivotal role in human skin carcinogenesis (Agar et al., 2004). At present, protection against solar UVA-induced oxidative damage is mainly depends on using sunscreens. However, the current sunscreens formulations possess rather protection against UVB rather than UVA (Haywood et al., 2003).

In this study, the cloning of a synthetic gene coding for CP6 was employed to induce function expression in *E. coli*. The expressed CP6 was about 46 kDa and the

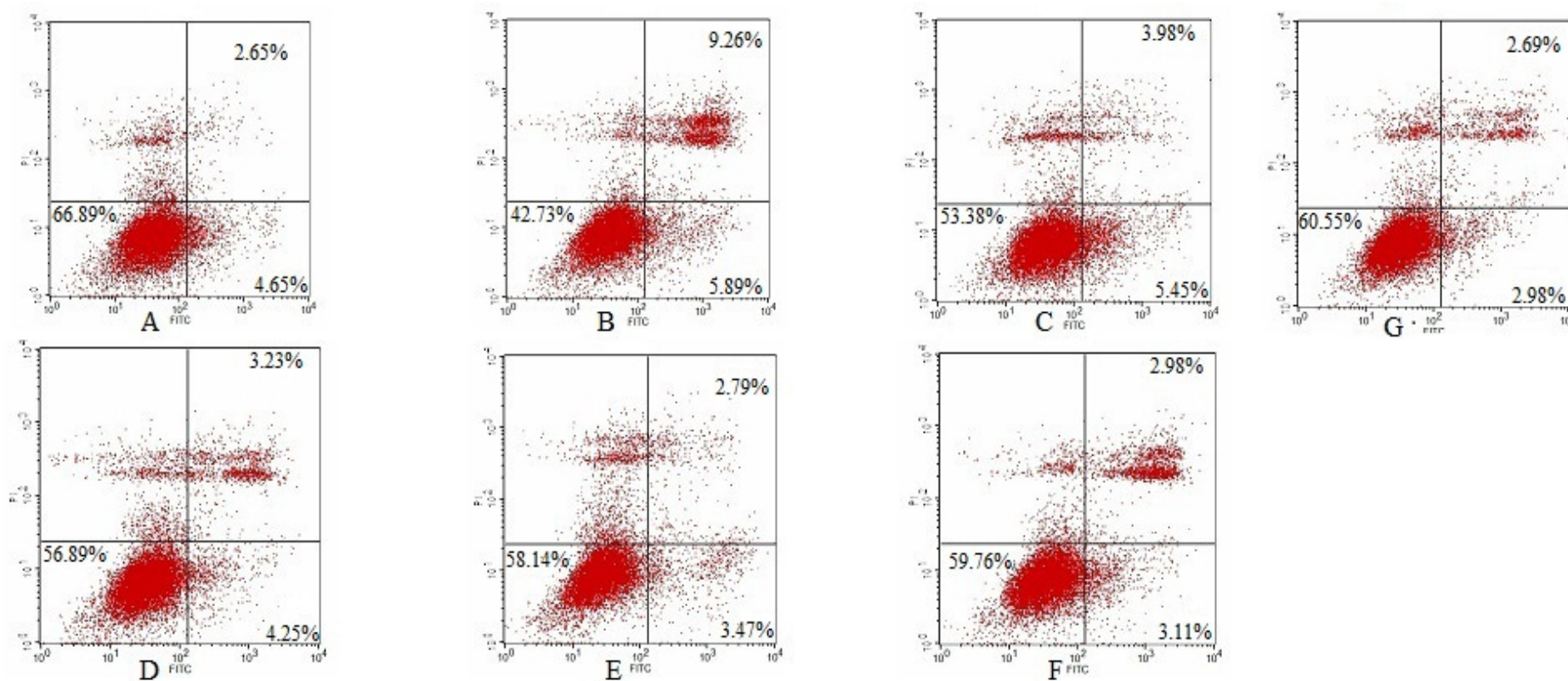


Figure 3. The results of HSF apoptosis by Annexin V/PI with flow cytometry. (a) Untreated control HSF cells. (b) Before adding CP6, UVA-treated cells showed apoptotic features. (c) CP6 (2 mg/mL) added before UVA-irradiation. (d) CP6 (4 mg/mL) added before UVA-irradiation. (e) CP6 (6 mg/mL) added before UVA-irradiation. (f) CP6 (8 mg/mL) added before UVA-irradiation. (g) CP6 (10 mg/mL) added before UVA-irradiation.

expression level was approximately 10% of the total bacteria protein. Fractionation using SDS-PAGE revealed that most CP6 existed in the soluble fraction (supernatant).

It is well known that UVA is able to induce keratinocyte apoptosis, so we established a model for UVA-damage. Detection of cell death in the presence of CP6 demonstrated a dose-dependent cytoprotective effect as revealed by MTT, while CP6 did not increase cell death in control cultures,

indicating that CP6 pre-treatment was safe and protective.

To further investigate the cytoprotective effect of CP6, we have measured programmed cell death using Annexin V-FITC/PI and TUNEL methods. Annexin V-FITC is a phospholipid-binding protein with a high affinity for phosphatidylserine (PS), which can be used as a sensitive probe for PS exposure to the cell membrane (Martin et al., 1995; Bartkowiak et al., 1999). Double staining of

the infected HSF with annexin V-FITC and PI in this research could distinguish apoptotic cells from necrotic cells (Yao et al., 2007; Chen et al., 2008). Cell death by apoptosis is characterized by DNA fragmentation in 200 to 250 and/or 30 to 50 kilobases.

Further, internucleosomal DNA fragmentation in 180 to 200 base pairs may also occur. Such characteristics have been used to distinguish apoptotic cells from normal or necrotic cells. To

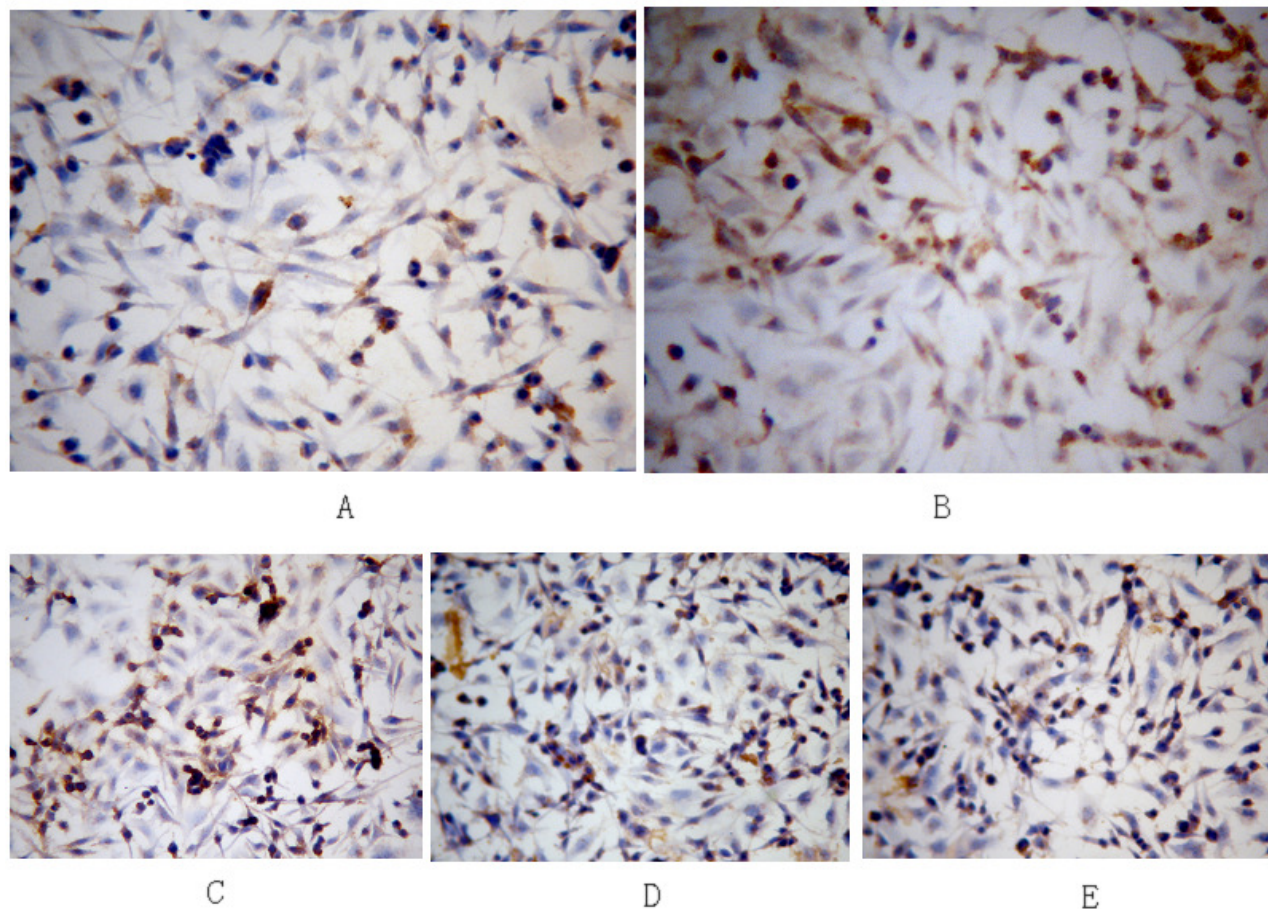


Figure 4. HSF apoptosis detected with TUNEL. (a) Untreated control HSF cells. (b) Before adding CP6, UVA-treated cells showed apoptotic features. (c) CP6 (2 mg/mL) added before UVA-irradiation. (d) CP6 (4 mg/mL) added before UVA-irradiation. (e) CP6 (6 mg/mL) added before UVA-irradiation. (f) CP6 (8 mg/mL) added before UVA-irradiation. (g) CP6 (10 mg/mL) added before UVA-irradiation.

detect apoptotic cells, whatever the pattern of DNA fragmentation, the TUNEL method is commonly utilized (Steensma et al., 2003; Huerta et al., 2007; Gautschi et al., 2009). In this study, the results suggested that CP6 can effectively suppress the apoptosis induced by UVA-irradiation.

Although, we have confirmed that CP6 has powerful antioxidant and protective activities against UV-induced damage abilities *in vitro*, its overall effectiveness in skin protection *in vivo* have yet to be elucidated. Studies focused on the development of novel agents with protective activities against UV-induced damage, particularly from recombinant proteins techniques in biotechnology research are intensively being carried out. In this study, we concentrated on the UVA part of UV light, whose relevance has been marginalized for a long period.

Conclusions

CP6 could improve the activities of proliferation and

decrease the apoptosis rate of HSF. However, skin is a complicated organ consisting of several layers and types of cells which influence each other during and after irradiation. Thus, further research is needed to specify the effect of CP6 on other skin cells, as well as to define CP6's effect and safety *in vivo* in animal models and humans.

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