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Full Length Research Paper

Expression of recombinant human collagen peptide in *Escherichia coli* and effects of the recombinant protein on UVA-irradiated HaCaT Cells

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In this study, we reported the cloning and over- expression of a gene encoding human collagen peptide (CP6) in *Escherichia coli* and the establishment of a purification protocol for obtaining the recombinant protein. The collagen peptide (CP6) had a molecular weight of about 46 kDa, and CP6 expression comprised approximately 10% of the total bacterial protein expression. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed that most of the collagen peptide (CP6) existed in the soluble fractions. Purified collagen peptide (CP6) was highly soluble. By using high-performance liquid chromatography (HPLC), a CP6 yield of approximately 11.4 mg/L of Luria-Bertani (LB) broth was obtained. What's more we performed assays with 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and annexin V-fluorescein isothiocyanate/propidium iodide (annexin V-FITC/PI) to investigate the cytoprotective effects of CP6 on the proliferation of UVA-damaged human keratinocyte cell line cells. The results of this study showed that CP6 could prevent UVA-induced DNA damage in cells of the spontaneously immortalized human keratinocyte cell line HaCaT.

Key words: Human collagen peptide, *Escherichia coli*, HaCaT, UVA.

INTRODUCTION

The generation of recombinant proteins is one of the most widely used techniques in biotechnology research (Makrides, 1996; De Anda et al., 2006). Expression of recombinant proteins in *Escherichia coli* has many advantages, including cost-effectiveness, easing of propagation, and the possibility of generating large amounts of proteins (Makrides, 1996; Baneyx, 1999; Swartz, 2001; Baneyx and Mujacic, 2004; Wong et al., 2008). Collagen is the most abundant protein in the vertebrate body, comprising about one-third of the total protein content of the body (Yi et al., 2005; Lebbink et al., 2006). Collagens are fibrous, extracellular matrix protein with high tensile strength (Wong and Kaplan, 2002; Franchi et al., 2007). The protein is a major component of connective tissues such as tendons and cartilage. There are several types of collagen protein, including fibril-forming interstitial collagen, basement membrane collagen, and beaded filament collagen (Chu et al., 1987; Heinegård, 2008).

Collagen alpha- 2(VI) acts as a cell- binding protein that is involved in the organization of the extracellular matrix (Chu et al., 1989; Paola et al., 2001). 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 (Saitta et al., 1992; Kuo et al., 1997; Lucarini et al., 2005; Lampe and Bushby, 2005; Merlini et al., 2008; Tanaka et al., 2009). The skin is constantly exposed to ambient ultraviolet (UV) radiation that can cause photo- oxidative damage and result in non-melanoma skin cancer, sunburn, and photo-aging, which is characterized by wrinkles and the loss of skin tone and resilience (Brash et al., 1991; Li et al., 1995; Wlaschek et al., 2001; Rass and Reichrath, 2008). The ultraviolet A (UVA) radiation component of sunlight is the most important environmental factor involved in the pathogenesis of skin cancer because it causes mutations, some of which may lead to malignant transformation (Setlow et al., 1993;

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Drobetsky et al., 1995; Stary et al., 1997; Setlow, 1999; You and Pfeifer, 2001; Cotten et al., 2003; Soufir et al., 2004).

In this study, we described the cloning and expression of the collagen peptide COL6A (or CP6) in *E. coli* and defined a purification protocol for the recombinant protein. 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl-tetrazolium bromide (MTT) assay and annexin V-fluorescein isothiocyanate/propidium iodide (annexin V-FITC/PI) were performed to investigate the cytoprotective effect of CP6 on the proliferation of UVA-damaged cells of a human keratinocyte cell line.

MATERIALS AND METHODS

Bacterial strains, plasmids and cells

All chemicals used were of analytical grade and were purchased from Jilin Chemical Co. (Changchun, China) unless otherwise stated. The restriction enzymes EcoRI and SalI, T4 DNA ligase, and Ex Taq were obtained from Sigma Chemical Co., USA. The DNA gel extraction, plasmid extraction, and polymerase chain reaction (PCR) product purification kits were purchased from Takara. *E. coli* (Invitrogen^{TM} by Life Technologies. USA) was used as the host strain for manipulation. *E. coli* BL21 (DE3) RIL (invitrogen) was used as the expression strain. The spontaneously immortalized human keratinocyte cell line HaCaT (ATCC) was used to examine the effect of CP6.

Expression plasmid construction and transformation of *E. coli*

To construct the expression plasmid, PCR was performed with genomic full-length human *COL6A2* (Origene Technologies) with the following sequences:

1F (5 -CCGGAATTCGGCAACAAAGGAGCCAAG-3), the forward primer sequence (F); 1R (5 -GCGTCGACGTTCTTGACAGCCTCCTT-3), the reverse primer sequence (R) (the under lined bases specify the EcoRI and SalI restriction sites in the F and R primers, respectively).

The PCR thermocycler protocol was as follows:

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, extension termination at 72°C for 1 min, and a single incubation at 72°C for 7 min. The PCR products were analyzed on 1.2% agarose gels, and the desired nucleotide band of about 700 bp (Figure 1A) was excised from the gel and purified by using a DNA extraction kit. The DNA was digested with EcoRI and SalI and ligated into a similarly digested pET-32a vector by using T4 DNA ligase.

The recombinant plasmid containing the *Homo sapien* collagen peptide gene *COL6A2* was named pET-32a-CP6. The pET-32a-CP6 plasmids were introduced into *E. coli* DE3. The pET-32a expression vector allows the expression of the recombinant proteins as N-terminal 6-His-tagged fusions.

Expression and purification of collagen peptide (CP6)

DE3, a genetically engineered microorganism, was cultured overnight with shaking at 37°C in LB medium containing ampicillin (100 g/mL). This overnight culture was used as the inoculum for a 250-mL flask of fresh LB. When the OD at 600 nm showed a reading of 0.6 to 0.7, protein expression was induced by the addition of isopropyl- -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 for 15 min at 4°C, and the cell pellets were washed and then re-suspended in 50 ml of ice-cold binding buffer (His·Bind Purification Kit.; Novagen®). Cells were placed on ice and lysed by sonication. The lysate was centrifuged at 12000 × g for 10 min at 4°C. The supernatant and the pellet containing the soluble and insoluble protein fractions, respectively, were then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (invitrogen). A protein band with size consistent to that of CP6 was detected in both the pellet and soluble fractions.

The isolated supernatant protein was subjected to high-performance liquid chromatography (HPLC) on a Ni- NTA His·Bind Superflow™ resin column (Novagen®), which had been preequilibrated with 1× Ni-NTA wash buffers (His·Bind Purification Kit., Novagen®). The proteins were eluted sequentially with a linear gradient of imidazole (60 to 300 mM). Finally, the bound proteins were eluted with 1× Ni-NTA elution buffer (Novagen®). After SDS-PAGE analysis, fractions containing nearly pure collagen peptide (CP6) were pooled.

SDS-PAGE and western blotting

To identify the obtained collagen peptide (CP6), western blotting was carried out by using the mouse anti-human collagen VI A2 antibody (Santa Cruz Inc.). Collagen peptide (CP6) samples were fractionated by SDS-PAGE, and CP6 was then transferred to nitrocellulose membranes. After being washed, the membranes were incubated with 1:2000 horseradish peroxidase- conjugated goat anti-mouse IgG (goat anti-mouse IgG-HRP) (Cruz Marker™). The stained membranes were developed by using the electrochemiluminescence (ECL) detection system according to the manufacturer's specifications (Figure 1C).

CP6 activity assay

Cultures of the spontaneously immortalized human keratinocyte cell line HaCaT (ATCC) were grown at 37°C in DMEM with 10% fetal calf serum in a humidified atmosphere containing 5% CO_2. The cells underwent different treatments:

(A) Cells were cultured to 80% confluence, various doses of CP6 (2, 4, 6, 8, and 10 mg/mL) were added to the culture, and the cells were exposed to UVA (5 mJ/cm^2). (B) Cells were cultured to 80% confluence, irradiated with UVA (5 mJ/cm^2), and cultured with various doses of CP6 (2, 4, 6, 8, and 10 mg/mL) for 12 h.

During irradiation, the medium was discarded and the cells were covered with 2 ml of phosphate buffered saline (PBS). Cell viability was assessed with the MTT assay. Briefly, 20 l of 5 mg/mL MTT dye solution was added to the medium. The cells were then incubated at 37°C for 4 h, and the medium was replaced with 150 l of dimethylsulfoxide. The absorbance was measured at 570 nm in a "microplate reader".

Apoptosis was quantified by using an annexin V-FITC detection kit (Abcam) in accordance with the manufacturer's instructions. The cells were washed twice with cold PBS, and the PBS was then gently removed from the culture surface. After being removed most of the PBS, the cell pellet was resuspended in 400 L of 1× binding buffer with 5 L of annexin V–FITC mixture and gently vortexed. Propidium iodide (10 L) was then added, and the cells were incubated for 5 min. Data were analyzed using the WinMDI software package.

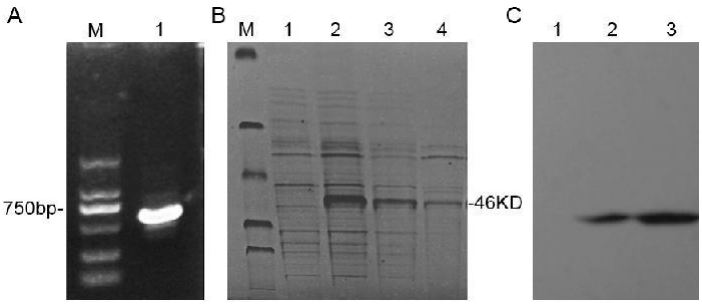

Figure 1. A) The PCR products of the target genes. Lane M: DNA marker; Lane 1: The PCR fragment of CP6. B) Expression of CP6 in *E. coli* BL21 (DE3). Lane M: Protein markers; Lane 1: Un-induced *E. coli* cell lysate; Lane 2: *E. coli* lysate after IPTG induction; Lane 3: Supernatant of the *E. coli* lysate after ultrasonication of the induced *E. coli*; Lane 4: Pellet fraction of the *E. coli* lysate after ultrasonication of the induced *E. coli*. C) 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*.

Statistical analysis

All data are presented as the mean± SD. The data were analyzed statistically by the SPSS ® software package (Version 11.01 d, SPSS GmbH Software, Munich, Germany). a *p*-value of less than 0.05 was considered significant.

RESULTS

Expression and purification

The plasmids were introduced into *E. coli* BL21 (DE3) and expressed. The expressed CP6 had a molecular weight of about 46 kDa, and CP6 comprised approximately 10% of the total bacterial protein by weight. SDS-PAGE analysis (Figure 1B) revealed that most target proteins existed in the soluble fractions (supernatant); few proteins were found in the insoluble protein fractions (precipitate). Purification of the recombinant protein was achieved by HPLC. The Ni- NTA His·Bind resin column was effective in enriching the His-tagged recombinant protein, which comprised more than 90% of the total protein eluted with imidazole. The purified recombinant protein had a purity of more than 95%, as revealed by Coomassie blue staining of the SDS-PAGE gels. Approximately 11.4 mg of recombinant protein was obtained from 1 L of bacterial culture in LB broth.

Cytoprotective effect of CP6 on UVA-irradiated HaCaT cells

The effect of various doses of CP6 on HaCaT cell viability was tested, and the results indicated that the recombinant protein had no toxicity. We established a UVA-damage model to investigate whether CP6 protected HaCaT cells from the apoptosis induced by UVA; the cells were then exposed to 5 mJ/cm^2 UVA in the following experiments. MTT assay and flow cytometric analysis were performed to evaluate the prophylactic effect of CP6 on HaCaT cells with UVA-induced damage. Data analysis showed that cell viability in the presence of CP6 was higher than that in the non-treated cells. The results were shown in Table 1 and Figure 2. They revealed that CP6 exerted a cytoprotective effect on the UVA-irradiated HaCaT cells.

Therapeutic effect of CP6 on UVA-irradiated HaCaT cells

MTT assay and flow cytometric analysis were performed to evaluate the proliferative effect of CP6 on HaCaT cells. Data analysis showed that cell proliferation was more rapid in the presence of CP6 (Table 1 and Figure 2). We found that CP6 promoted the proliferation of UVA-damaged HaCaT cells. The effect of CP6 on UVA-damaged HaCaT cells was not only prophylactic but also therapeutic, but the prophylactic effect was greater than the therapeutic effect.

DISCUSSION

This review discussed the use of recombinant microbial expression systems as a cost-effective, scalable production technology for manufacturing a novel biomaterial with consistent quality and improved safety: recombinant human collagen peptide. Recombinant

Table 1. Effect of CP6 on UVA-induced HaCaT cells.

CP6 added after UVA-irradiation		CP6 added before UVA-irradiation	
A	0.85783 ± 0.016055	A1	0.81267 ± 0.009352
B	0.43183 ± 0.010962	B1	0.4385 ± 0.032017
C	0.4445 ± 0.018338	C1	0.5065 ± 0.014324*
D	0.49967 ± 0.008756	D1	0.5545 ± 0.013248*
E	0.53103 ± 0.013512*	E1	0.57333 ± 0.013186*
F	0.56522 ± 0.036149*	F1	0.65483 ± 0.019874*
G	0.5805 ± 0.030422*	G1	0.70617 ± 0.006306*

(A) Untreated control HaCaT cells. (B) UVA-treated cells showed apoptotic features without CP6. (C) CP6 (2 mg/mL) added after UVA-irradiation. (D) CP6 (4 mg/mL) added after UVA-irradiation. (E) CP6 (6 mg/mL) added after UVA-irradiation. (F) CP6 (8 mg/mL) added after UVA-irradiation. (G) CP6 (10 mg/mL) added after UVA-irradiation. (A1) Untreated control HaCaT cells. (B1) UVA-treated cells showed apoptotic features without CP6. (C1) CP6 (2 mg/mL) added before UVA-irradiation. (D1) CP6 (4 mg/mL) added before UVA-irradiation. (E1) CP6 (6 mg/mL) added before UVA-irradiation. (F1) CP6 (8 mg/mL) added before UVA-irradiation. (G1) CP6 (10 mg/mL) added before UVA-irradiation. *P < 0.01 from UVA-irradiated group before addition of CP6.

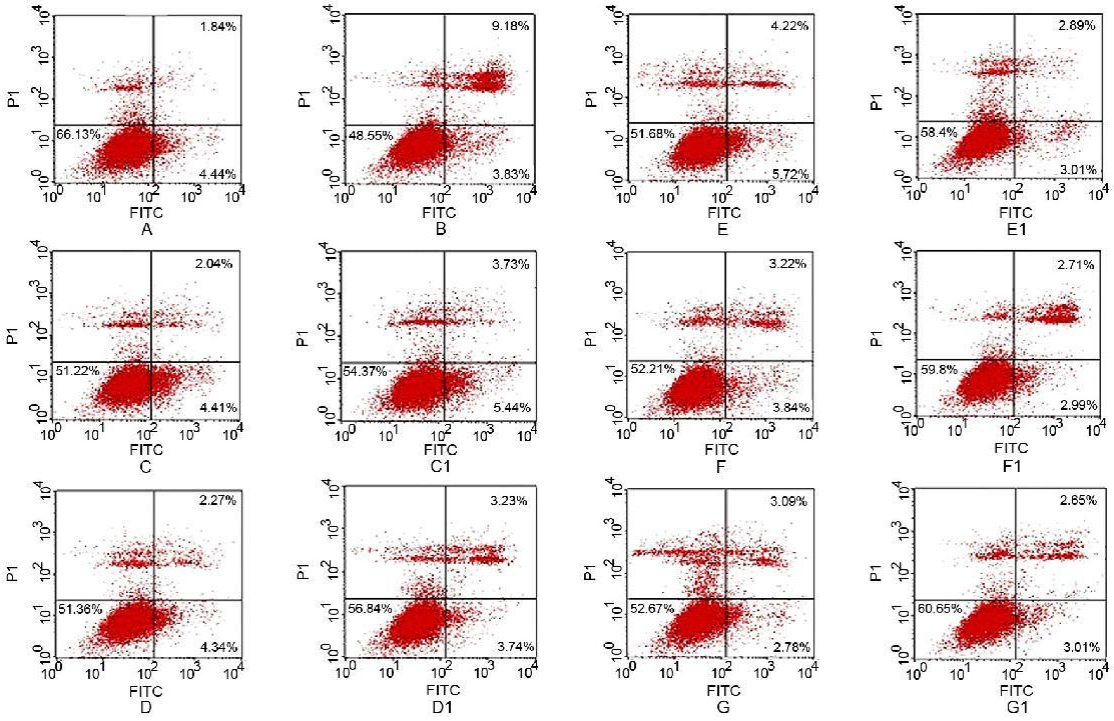

Figure 2. Effect of CP6 on HaCaT cells with UVA-induced damage. (A) Untreated control HaCaT cells. (B) Before adding CP6, UVA-treated cells showed apoptotic features. (C) CP6 (2 mg/mL) added after UVA-irradiation. (D) CP6 (4 mg/mL) added after UVA-irradiation. (E) CP6 (6 mg/mL) added after UVA-irradiation. (F) CP6 (8 mg/mL) added after UVA-irradiation. (G) CP6 (10 mg/mL) added after UVA-irradiation. (C1) CP6 (2 mg/mL) added before UVA-irradiation. (D1) CP6 (4 mg/mL) added before UVA-irradiation. (E1) CP6 (6 mg/mL) added before UVA-irradiation. (F1) CP6 (8 mg/mL) added before UVA-irradiation. (G1) CP6 (10 mg/mL) added before UVA-irradiation.

microbial technology has been proposed as an approach for the production of other important protein-based biopolymers such as elastin and dragline spider silk (Báez et al., 2005; Du et al., 2008). Some of these biopolymers can only be commercialized by using recombinant technology (silk and gelatin as defined collagen fragments); in other cases, recombinant technology provides attractive substitutes for animal and human tissue-derived biomaterials, especially in pharmaceutical applications (Olsen et al., 2003). The technology also enables the engineering of biopolymers to improve the performance of products containing these

biomaterials.

In this study, a gene encoding CP6 was cloned into *E. coli* cells to induce functional expression of CP6 in *E. coli*. The expressed CP6 had a molecular weight of about 46 kDa and the expressed protein comprised approximately 10% of the total bacterial protein. Fractionation using SDS-PAGE revealed that most of the CP6 existing in the soluble fraction (supernatant) . *E. coli* proved to be an effective production system for the manufacture of recombinant human collagen peptide. UVA can induce keratinocyte apoptosis (Denning et al., 1998; Assefa et al., 2005). Therefore, we established a UVA-damage model to investigate the effect of CP6. We performed MTT assays and flow cytometric analysis to investigate the cytoprotective and therapeutic effect of CP6 on UVA-damaged HaCaT cells. We found that the effect of CP6 on UVA- damaged HaCaT cells is not only prophylactic but also therapeutic, but the prophylactic effect was greater than the therapeutic effect. The mechanisms by which CP6 confers cytoprotection and enhances proliferation remain unclear. For instance, we have not yet determined whether CP6 acts intracellularly or extracellularly, or both.

Further studies are clearly needed to elucidate the cytoprotective actions of the collagen peptide (CP6).

Conclusions

In conclusion, the present results suggest that the collagen peptide (CP6) had a molecular weight of about 46 kDa, and CP6 expression comprised approximately 10% of the total bacterial protein expression. Most of CP6 existed in the soluble fractions and a CP6 yield of approximately 11.4 mg/L of Luria-Bertani (LB) broth was obtained. Besides, CP6 could prevent UVA-induced DNA damage in cells of the spontaneously immortalized human keratinocyte cell line HaCaT.

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