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Protective effects of κ-ca3000 + CP against ultraviolet-induced damage in HaCaT and MEF cells

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ABSTRACT

In this study, the complex κ-ca3000 + CP combined collagen peptide with κ-carrageenan oligosaccharide was tested for its ability to moderate UV-induced damage and investigated for its protective mechanism against UV radiation. Human keratinocytes (HaCaT) and mouse embryonic fibroblasts (MEF) were used to monitor the effects of κ-ca3000 + CP on cell viability, apoptosis, level of collagen I and MMP-1, MAPKs activation and intracellular ROS production after UV-irradiation. The results indicated that application of the κ-ca3000 + CP (100 μg/ml) could significantly attenuate UV-induced HaCaT and MEF death, as well as inhibit the UV-induced apoptosis of HaCaT cells. The decreased collagen I synthesis and the increased MMP-1 expression of MEF by UV radiation were almost restored back to normal level after treatment with κ-ca3000 + CP. Moreover, κ-ca3000 + CP could significantly suppress UV-induced MAPKs activation and intracellular ROS production. Taken together, these results showed that antioxidant property of κ-ca3000 + CP can effectively attenuate UV-caused cell damage and skin photoaging by suppressing cell apoptosis and expression of MMP-1 through the MAPKs signaling pathways. Thus, κ-ca3000 + CP has potential antiaging effects and prominent protective effects on UV-induced skin cell damages, which might be used in pharmaceutical and cosmetic industries.

1. Introduction

High dose of UV-irradiation is often known to be one of the most ubiquitous environmental hazards that impact every living creature under the sun. Skin is the largest human organ, and is the only organ directly exposed to UV-irradiation [1,2]. A growing body of evidence suggests that reactive oxygen species (ROS) are generated by UV radiation, especially for UVB (290–320 nm) and UVA (315–400 nm) radiation, resulting in oxidative damage to both epidermal and dermal cells [2–4]. Severe oxidative stress may result in photoaging, and even apoptotic or necrotic skin cell death [3–5]. Thus, application of non-toxic and high-performance antioxidants could be a successful strategy for protecting the skin against ROS-induced injury [6].

Oceans are important natural resources for several peptides and oligosaccharides with potential antioxidant properties. In our previous study, κ-carrageenan oligosaccharides with high contents of sulphate groups showed strong antioxidant activities, especially for the oligosaccharides with molecular weight of about 3 kDa (κ-ca3000). Hou et al. [7] reported the bioactive tilapia skin collagen peptides (CP) rich in hydroxyl and carboxyl groups exhibited antioxidant activities and protective functions against ultraviolet radiation (UV) induced skin ailments. The specific properties of each peptide and oligosaccharide from marine active extracts offer possibilities to produce complexes that can confer unique novel properties [8]. Thus, the scavenging free radical ability and the protective effects of the peptide–oligosaccharide complex κ-ca3000 + CP against UV radiation have caused our concerns and interests.

In this study, we prepared the collagen peptide–κ-carrageenan oligosaccharide complex κ-ca3000 + CP and tested its ability to moderate photodamage, with the investigation of its protective mechanism against UV-irradiation.

2. Materials and methods

2.1. Reagents

3-(4,5-Dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma–Aldrich (USA). Dulbecco's Modified Eagle Medium (DMEM), Modified Eagle's Medium (MEM), penicillin, streptomycin, and 0.25% trypsin were purchased from Gibco (USA). All other reagents used were of analytical grade.

κ-Carrageenan was kindly provided by School of Medicine and Pharmacy, Ocean University of China. Bioactive tilapia skin

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collagen peptide was generously provided by Chinese Center of Marine Biotechnology/BAC/UNESCO.

2.2. Preparation of peptide–oligosaccharide complex

2.2.1. Isolation and purification of the κ-carrageenan oligosaccharides

The fraction of κ-carrageenan oligosaccharides (degree of polymerization (DP) = 7–19), namely κ-ca3000, was prepared by acid hydrolysis of the 1% (w/v) corresponding block solution in 0.1 M H_2SO_4 at 60 °C for 1 h, and was neutralized by $BaCO_3$ [9]. The obtained oligosaccharides were then size-fractionated by preparative gel filtration chromatography on a 100 cm × 1.5 cm Bio-Gel P6 column followed by elution with 0.1 M NH_4HCO_3 at a flow rate of 15 ml/h [10]. The target oligosaccharides were collected in terms of degree of polymerization (DP = 7–19) and immediately freeze-dried.

2.2.2. Determination of molecular weight

The average molecular weight of the collected fraction was measured by high performance liquid chromatography (HPLC) according to Yamamoto et al. [11] with TSK-G2000 SW column (7.5 mm × 300 mm, Japan) on Agilent 1100 LC equipped with refractive index detector (RID). The system was maintained at 35 °C and the mobile phase was 0.1 M Na_2SO_4 at a flow rate of 0.1 ml/min. The collected fraction was dissolved in 0.1 M Na_2SO_4 to a final concentration of 10 mg/ml and was filtered through 0.22 μm filter membrane before analysis. The column calibration was performed with various dextran standards (Mw: 180, 1200, 2500, 4600, 7100, 10,000, and 11,700 Da) purchased from Fluka. Molecular weight of the collected fraction was calculated using the Angilent GPC software (USA).

2.2.3. Cross-linking

The standardized oligosaccharide fraction (κ-ca3000) and the bioactive tilapia skin collagen peptide (CP) were separately dissolved in double-distilled water at a concentration of 4% (w/v) and heated at 50 °C for 30 min while continuously stirring. Subsequently, the κ-ca3000 and CP were mixed in the proportion of 1:1 in order to obtain the peptide–oligosaccharide complex, namely κ-ca3000 + CP. The blended solution was stirred for 1 h at 50 °C for homogeneity and then freeze-dried [12,13].

2.3. Cell culture

Mouse embryonic fibroblast (MEF), from Kunming white foetus, was isolated according to the method of Xue [14], and cultured in 5% CO_2 at 37 °C in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Generation 2–5 of MEF cells were selected for further assays.

Human keratinocyte cell line (HaCaT), purchased from China Center for Type Culture Collection (CCTCC), was cultured in MEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and maintained at 37 °C with 5% CO_2 in a humidified atmosphere.

2.4. UV-irradiation and treatment

The cells were randomly divided into different groups including control group (normal cultural cells), model group (UV-irradiated cells), compound group (different compound pretreated and UV-irradiated cells). When grown to 80–90% confluence, cells were subjected to irradiation with both UVA lamps (320–400 nm) and UVB lamps (290–320 nm) (Beijing Normal University, China) at a dose of 5 J/cm^2 and 15 mJ/cm^2 [15], which were monitored by UVA and UVB radiometers (Beijing Normal University, China) respectively.

Each compound was separately added to the cell culture medium 1 h before irradiation at different final concentrations (10, 20, 50, and 100 μg/ml). Then the mixtures were aspirated and the cells were covered with PBS during irradiation. After irradiation, PBS was replaced with the original medium and the cells were incubated for different periods at 37 °C in 5% CO_2 [16].

2.5. Cell viability assays

Cell viability was evaluated by the MTT colorimetric assay as described by Alesiani et al. [17]. MTT was dissolved in PBS at 5 mg/ml. Cells were seeded in 96-well microplate (5×10^4 cells/ml) 24 h, and then were treated according to the method described in Section 2.4. After incubation for 18 h, the cells were treated with MTT for 4 h at 37 °C in 5% CO_2. After that, the medium with MTT was removed and 100 μl of dimethylsulfoxide (DMSO) was added to cells. The absorbance was measured at 490 nm in an automatic microplate photometer.

In addition, we also evaluated the effects of κ-ca3000 + CP treatment after UV-irradiation on cell viability. Prior to UV-irradiation, cells were seeded in 96-well microplate (5×10^4 cells/ml) 24 h, and then washed and covered with PBS. After UV-irradiation PBS was removed, serum free medium with each compound (10, 20, 50 and 100 μg/ml) was applied and the cells were incubated thereafter at 37 °C for 18 h. After that, the cell viability was measured by MTT method as described above.

2.6. Modulation of UV-induced apoptosis by κ-ca3000 + CP

2.6.1. Flow cytometric analysis of cell cycle

Apoptotic cells show a diminished staining below the G0/G1 population of normal diploid cells. The DNA specific fluorochrome Propidium Iodide (PI) can identify a distinct hypo-diploid cell population, which was measured according to the method of Vermes et al. [18]. After UV-irradiation and then incubation for 18 h [16], 1.0×10^6 HaCaT cells were trypsinized and washed with PBS by centrifuging (200*g*). After that, cellular DNA was stained with the PI-staining solution (PI 50 pg/ml in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma) in polypropylene tubes for 15 min at room temperature in darkness. The distribution of cells in the different cell cycle phases was analyzed from the DNA histogram using a Bectone Dickinson FACS Vantage flow cytometer and Cell Quest software samples [19].

2.6.2. TUNEL assay

DNA-fragmentation in HaCaT cells was evaluated by TdT-mediated dUTP nick end labeling (TUNEL) assay [20]. After UV-irradiation and then incubation for 2 h [21], the apoptosis of cell samples was measured by TUNEL staining according to the manufacturer's protocol of DeadEnd™ Fluorometric TUNEL assay (Promega, USA), and combined with PI-staining for cell nuclei. The green fluorescence of Fluorescein-12-UTP and the red fluorescence of PI were measured at 520 ± 20 nm and at >620 nm respectively by Laser Scanning Confocal Microscope (Zeiss LSM 510, GER).

2.6.3. Cleavage of PARP

After reaching confluence, the HaCaT cells were trypsinized and then plated on a 6-well culture dish at 5×10^5 cells per well in 1 ml of MEM containing 10% FCS. After 24 h, the medium was replaced with 1 ml of serum-free MEM containing the maximum efficient concentration of κ-ca3000 + CP, which was prescreened by the cell viability assays as described in Section 2.5. Following UV-irradiation, cell samples were incubated for 12 h [22], and then lysed in cell lysis buffer (62.5 mM Tris–HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.1% w/v bromophenol blue). Equal amounts of the protein samples were subjected to 10% SDS–PAGE and the