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The Level of UVB-induced DNA Damage and Chemoprevention Effect of *Paeoniflorin* in Normal Human Epidermal Kerationcytes

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Abstract

Ultraviolet (UV) radiation to mammalian skin is known to alter cellular function via generation of Reactive Oxygen Species (ROS), DNA damage and DNA lesions, such as pyrimidine dimmers and photoproducts, which could lead to DNA mutation if they are not repaired. In this study, we have investigated the reduction of DNA damage and of apoptosis with a particular attention to genetic effect of paeoniflorin in Normal Human Epidermal Keratinocytes (NHEK). After UVB irradiation from 10 to 500 mJ/cm^2 to NHEK, Mean Tail Moments (MTM) were increased with UVB dose increase. The greatest amount of strand breaks was induced at 500 mJ/cm^2 of UVB. Even at the lowest dose of UVB (10 mJ/cm^2), change in MTM was detected ($P < 0.0001$). Pretreated cell with 0.1% paeoniflorin maximally reduced the level of DNA damage to about 21.3%, compared to untreated cell. In the lower concentrations less than 0.01% of paeoniflorin, MTM had a small increase but paeoniflorin still had reductive effects of DNA damage. We measured the apoptosis suppression of paeoniflorin with annexin V flous staining kit. As we observed under the fluorescence microscopy to detect apoptosis in the irradiated cell, the fluorescence intensity was clearly increased in the untreated cell, but decreased in treated cells with paeoniflorin. These results suggest that paeoniflorin reduces the alteration of cell membranes and prevents DNA damage. Therefore, the use of paeoniflorin as a free radical scavenger to reduce the harmful effects of UV lights such as chronic skin damage, wrinkling and skin cancer can be useful to prevent the formation of photooxidants that result in radical damage.

Keywords: paeoniflorin, ultraviolet, Normal Human Epidermal Keratinocyte (NHEK), single cell gel electrophoresis assay, DNA damage

Ultraviolet (UV) radiation is composed of UVA (320-400 nm), UVB (280-320) and UVC (200-280). UVC is effectively blocked by the ozone layer, but UVA and UVB radiation are reached at the Earth's surface of sufficient amount to have harmful biological effects to the skin. In particular, wavelengths in the UVB region of the solar spectrum are absorbed into the skin, producing the erythema, sunburns and wrinkles. Exposure of mammalian skin to UV light increases the cellular levels of Reactive Oxygen Species (ROS), which impair subcellular molecules such as nucleic acids, proteins and lipids in epidermal cells. The increase of ROS in cell resulted the loss of viability and the increase in membrane blebbing[1], cytoskeletal molecular changes[2-5] and apoptosis[6] and induce DNA lesions, such as pyrimidine dimmers and photoproducts, which could lead to DNA mutation if they are not repaired. To prevent DNA mutations, all mammalian cells were equipped with several DNA repair system, which are able to protect the cell from the effects of DNA damaging compounds by DNA lesions[7]. Depending upon the primary DNA lesion, one or more repair pathways became active, such as direct repair, base excision repair, mismatch repair, double stranded break repair and Nucleotide Excision Repair (NER). Among these repair systems, cyclobutane dimmers (CPDs) and photoproducts generated by UV irradiation were primarily repaired by NER, which removes bulky DNA damage in subpathways[8].

Peony is an ancient, traditional Chinese herbal medicine that provided useful medicine and attractive ornamental flowers for over 3,000 years in China and at least 500 years in Europe[9]. Peony root has been used to treat wounds, fungal infections, pain and spasmodic conditions in traditional Chinese medicine. Since paeoniflorin was isolated from the root of white paeony in 1969[10], Paeoniflorin has received growing research attentions for the pharmacological

effects; anti coagulant[11], neuromuscular blocking[12], cognition enhancement[13], and antihyperglycemic effects[14].

Okubo *et al.* showed that paeoniflorin has radical scavenging activity on DPPH radical and inhibitory effect on oxidative DNA cleavages[15]. However, Okubo's study only carried out the reaction of DNA with chemical in test tube, not in cell level. Therefore, DNA protecting effect of paeoniflorin still remains to be demonstrated to prevent the genetic toxicity in cellular level.

In this study, we have investigated the reduction of DNA damage and of apoptosis using a single cell gel electrophoresis, which is known as comet assay and a fluorescence detection method for phosphatidylserine as the marker of apoptosis to identify the antioxidant effects of paeoniflorin in a wide variety of systems in NHEK and a particular attention to genetic effect of paeoniflorin.

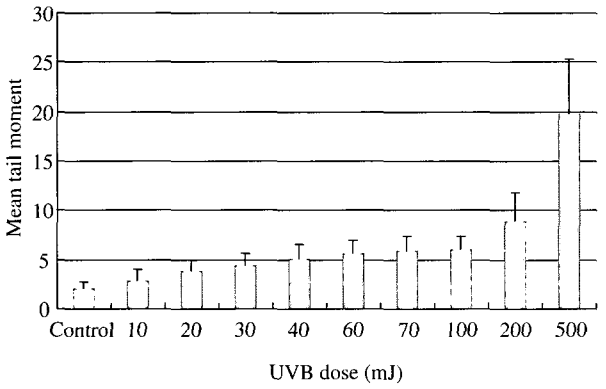

Fig. 1. Dose response of normal human epidermal keratinocytes to UVB irradiation as measured by the comet assay. Tail moment were analyzed by using CASP image program. Each point represents the mean tail moment from at least 50 comets measured in three individual experiments. Tail moments of treated cell was compared with that of untreated cell using student's *t*-test (***$P < 0.001$, control vs 10 mJ/ cm^2).

UVB Dose Response of Normal Human Epidermal Keratinocyte (NHEK) Measured using the Comet Assay

Cultures of NHEK were subjected to irradiation of UVB at the range of doses from 10 to 500 mJ/cm^2. After UVB irradiation, cells were prepared for analysis using a single cell gel electrophoresis (SCGE, comet assay) to determine DNA damage.

The result of the comet assay using the NHK is shown in Fig. 1. Mean Tail Moments (MTM) were increased with UVB dose increase. Exposure to UVB at 40 mJ/cm^2 caused a significant increase in DNA strand breaks ($P < 0.0001$), as can be seen from the greater DNA content in the comet tails of UV exposed cells as compared to control cells (Fig. 1). The greatest amount of strand breaks was induced at 500 mJ/cm^2 of UVB (Fig. 2). Even at the lowest dose of UVB (10 mJ/cm^2), we were able to detect change in MTM ($P < 0.0001$). These DNA strand breaks, if not repaired correctly, may cause gene mutation and skin carcinogenesis. Therefore, antioxidants are necessary to prevent both the physiological and the genetical damage.

Paeoniflorin Protects Against UV B Induced DNA Damage on NHEK

To identify the chemoprevention effects of paeoniflorin, we examined the reduction of UVB induced DNA damage in NHEK using comet assay. Firstly, we treated cell with 1% paeoniflorin, but not irradiated with UVB and carried out the comet assay. Paeoniflorin did not increase the tail moment in unstressed cell. We identified that paeoniflorin itself did not induce genetoxicity (Fig. 3). The majority of nuclei in non-UVB exposed samples as control were compact, round with only few cells showing DNA fragment moving, but in the negative control, irradiated cell had long tail and induced significant DNA damage. Pretreated cell with 0.1% paeoniflorin maximally reduced the level of DNA damage to about 21.3%, compared to untreated cell. In lower concentrations

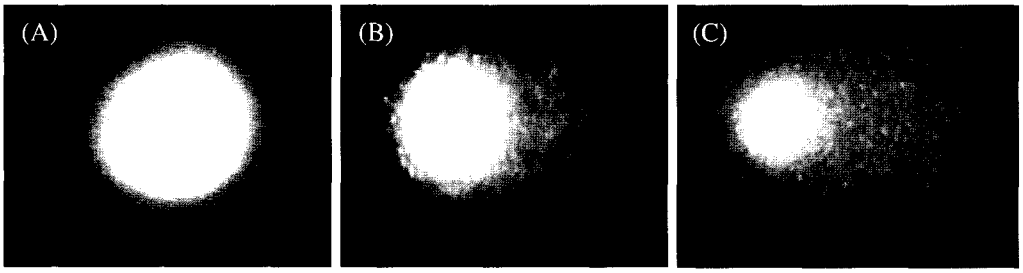

Fig. 2. Representative comets of normal human epidermal kerationcyte (NHEK) after exposure to (A) Non-treated, (B) UVB 60 mJ/cm^2 and (C) 500 mJ/cm^2.

less than 0.01% paeoniflorin, MTM had a small increase but paeoniflorin still had reductive effects of DNA damage (Fig. 3).

There were significant differences for reduction of tail moment between untreated cell and treated cell with 1% to 0.01% paeoniflorin. The result indicated that paeoniflorin has protective effects for DNA damage.

Monitoring Suppression of UV B Induced Apoptosis by Fluorescence Microscopy

The apoptosis suppression of paeoniflorin on NHEK treated at UVB 60 mJ/cm^2 were studied by measuring UV induced apoptosis with annexin V flous staining kit, 12 h after paeoniflorin treatment at different concentrations. As observed under light transmission microscopy, both untreated and treated cells to UV B did not show the morphology change compared with non irradiated cell (Fig. 4A, C). However, As observed under the fluorescence microscopy to detect apoptosis in the irradiated cell, the fluorescence intensity was clearly increased in untreated cells, but decreases in treated cells with paeoniflorin (Fig. 4B, D). This result indicated that paeoniflorin was decreased as the level of apoptosis induced by UV.

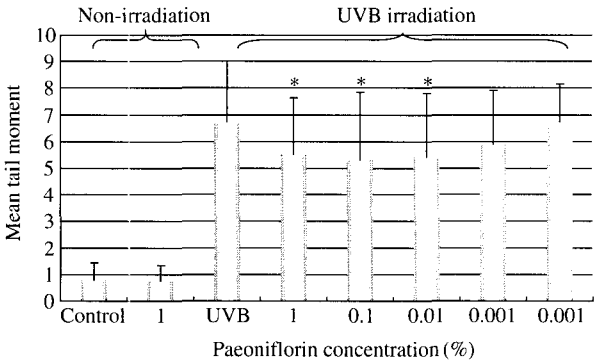

Fig. 3. Prevention of UVB induced DNA damage on primary humar keratinocytes treated with paeoniflorin. Pre treated human keratinocyte with paeoniflorin were irradiated with UVB (60 mJ/cm^2). Control was not irradiated but negative control was irradiated in untreated cell. Tail moment were analyzed by using CASP image program. Each bar represents the mean of at least three individual experiments. Tail moments of treated cell was compared with that of untreated cell using student's *t*-test (*$P < 0.05$).

In early apoptosis, phosphatidylserine on the outer leaflet of the plasma membrane is exposed[16], we detected apoptosis by using annexin V, a member of a family of proteins that bind to acidic phospholipids. We found that paeoniflorin decreased level of apoptosis at 1% paeoniflorin treated concentration and identified the antioxidant effects of paeoniflorin. The greatest effect of paeoniflorin is maintenance of cell morphology, protection of DNA damage and reduce of apoptosis in UVB irradiated cell.

These results suggest that paeoniflorin reduces the alteration of cell membranes and prevents DNA damage. Therefore, the use of paeoniflorin as a free radical scavenger to reduce the harmful effects of UV light such as chronic skin damage, wrinkling and skin cancer can be useful to prevent the formation of photo-oxidants that result in radical damage.

It is not clear exactly how paeoniflorin reduce UVB-induced apoptosis and DNA damage. Future studies will be processed in the mechanism by which paeoniflorin minimizes the apoptosis and DNA damage.

Methods

Chemicals and Reagents

All chemicals and organic solvents were from Sigma (St. Louis, MI, USA), unless otherwise in-

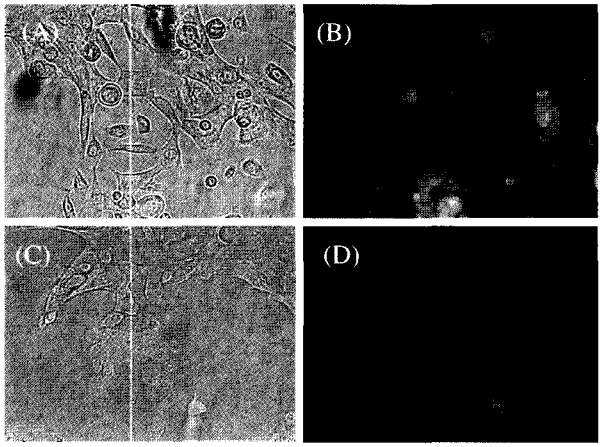

Fig. 4. Reduction of UVB induced apoptosis in normal human epidermal keratinocytes (NHEK) treated with 1% paeoniflorin. (A) NHEK by exposure to UVB (60 mJ/cm^2) was examined under the optical microscope, (B) Phosphatidylserine (PS) as a apoptosis marker was detected under the fluorescence microscope using annexin v flous staining kit (Loche), (C) and (D) apoptosis was reduced in treated cell with 1% paeoniflorin under the same condition.

dicated. Keratinocyte basal medium (KBM-2) and growth supplements were obtained from Cambrex (walkersvile, MD, USA). Trypsin-EDTA was purchased from GIBCO-BRL (Grand Land, NY, USA) and Diaspase II from Boheringer Mannheim (GmbH, German)

Preparation of Paeoniflorin

Paeoniflorin was extracted and partially purified from roots of *Paeonia lactiflora*. Briefly, peony root was grinded and extracted with 75% ethanol. The extracts concentrated by evaporation and resuspended in distilled water and fractionated with ethyl ether. The water soluble part was loaded to silica gel column and fraction containing peoniflorin was isolated with chloroform acetone (4 : 1) as moving phase. The purity of paeoniflorin was identified to about 64% by HPLC

Primary Keratinocyte Culture and Treatment

The adult foreskin samples were incubated overnight in 0.1% dispase/Dulbecco's modified Eagle's medium. The epidermis was removed and dispersed in 0.05% trypsin EDTA for 15 min. Cells were then plated out on fibronectin coated plates and grown in keratinocyte basal medium (KBM-2) mixed in a growth medium containing penicillin-streptomycin. After two passages the cultures were composed predominantly (>95%) of keratinocytes. The cells were used at passage 3 or 4. Cells were grown to 80% confluence, trypsinized with 0.05% trypsin EDTA, and plated at 60% in 60 mm dishes for experimental use (Becton Dickinson. Franklin Lakes, NJ. USA). Cells were cultured in keratinocyte growth medium at each of concentrations of paeoniflorin for 12 h. After 12 h incubation, Paeoniflorin containing medium were removed and replaced with phosphate-buffered saline (PBS) minus calcium and magnesium. Cells were irradiated in PBS with a solar simulator (PSI, Suwon, Korea). The 1-kW solar-simulating xenon arc lamp (Hanovia, Newwark, NJ, USA) was adjusted to a dose rate of 60 mJ per cm^2 and metered at 285 ± 5 nm with a research radiometer fitted with a UVB probe (model IL 1700 A; international Light, Newburyport, MA).

Comet Assay

Comet assay to detect DNA damage was performed under alkaline conditions essentially according to the procedure of Singh *et al.*[17]. The slides for comet assay were observed at 250 × magnification in a Metallux-3 fluorescence microscope (Leitz, German) attached to a color CCTV camera (Panasonic, Japan) and connected to a personal computer-based image analysis CASP downloaded from http://www.casp.of.pl. Fifty image were randomly selected from each sample and comet tail moment was measured, according to the procedure of Konca *et al.*[18]. The comet tail moment is positively correlated with level of DNA breakage. The mean value of the tail moment in a particular sample was taken as an index of DNA damage in this sample.

Determination of Apoptosis using Annexin V Fluorescence

Apotosis was determined by measuring Phosphatidylserine (PS) on the outer leaflet of apoptotic cell membrane using annexin-V-fluos staining kit (Roche. Penzberg, Germany) according to manufacture's directions and visualized under a fluorescence microscope (Leica, Wetzlar, Germany).

Statistics

All the values in this study were expressed as means ± S.D. using student's *t*-test. The level of statistical significance was taken as $P < 0.05$.

References

1. Malorni, W. *et al.* Both UVA and UVB induce cytoskeleton-dependent surface blebbing in epidermoid cells. *J Photo Chem Photobiol B:Biol.* **26**, 265-270 (1994).
2. Zamansky, G.B., Perrino, G.B. & Chou, I.N. Disruption of cytoplasmic microtubules by ultraviolet radiation. *Exp. Cell. Res.* **195**, 269-273 (1991).
3. Zamarsky G.B. & Chou, I.N. Disruption of keratin intermediate filaments by ultraviolet radiation in cultured human keratinocytes. *Photochem. Photobio.* **52**, 903-906 (1990).
4. Zamansky, G.B. & Chou, I.N. Environmental wavelengths of ultraviolet light induce cytoskeletal damage. *J. Invest. Dermatol.* **89**, 603-606 (1987).
5. Moll, I., Bohnert, E., Treib, U. & Jung, E.D. Effects of ultraviolet B radiation on cytoskeletal and adhesion molecules in human epidermis. *Photodermatol Photoimmunol. Photomed.* **10**, 26-32 (1994).
6. Godar, D.E. Preprogrammed and programmed cell death mechanisms of apoptosis: UV-induced immediate and delayed apoptosis. *Photochem. Photobio.* **63**, 825-830 (1996).
7. Wood, R.D. DNA repair in eukaryotes. *Annu. Rev. Biochem.* **65**, 135-167 (1996).
8. Lehmann, A.R. Nucleotide excision repair and the link with transcription. *Trends Biochem. Sci.* **20**, 402-405 (1995).
9. Fpster, S. & Yue, C.X. Herbal Emissries: *Bringing Chinese Herbs to the West.* Rochester, VT : Healing Arts Press. 200-207 (1992).

10. Aimi, N., Inaba, M., Watanabe, M. & Shibata, S. Chemical studies on the oriental plant drugs. 23. Peoniflorin, a glucoside of Chinese paeony root. *Tetrahedron.* **25**, 1825-1838 (1969).
11. Ishida, H., Takamatsu, M., Tusji, K. & Kosuge, T. Studies on active substances in herbs used for oketsu ("stagnant blood") in Chinese medicine. VI. On the anticoagulative principle in paeoniae radix. *Chem. Pharm Bulll.* **35**, 849-852 (1987).
12. Dezaki, K., Kimura, I., Miyahara, K. & Kimura, M., Complementary effects of paeoniflorin and glycyrrhizin on intracellular Ca^{2+} mobilization in the nerve-stimulated skeletal muscle of mice Jpn. *J. Pharmcol.* **69**, 281-284 (1995).
13. Watanabe, H. Candidates for cognitive enhancer extracted from medicinal plants: paeoniflorin and tetramethylpyrazine. *Behav. Brain. Res.* **83**, 135-141 (1997).
14. Hsu, F.L., Lai, C.W. & Cheng, J.T. Antihyperglycemic effects of paeoniflorin and 8-debenzoylpaeoniflorin, glucosides from the root of Paeonia lactiflora. *Planta. Med.* **63**, 323-325 (1997).
15. Okubo, T. *et al.* The inhibition of phenylhydroquinone-induced oxidative DNA cleavage by constituents of Moutan Cortex and Paeoniae Radix. *Biol. Pharm. Bull.* **23**, 199-203 (2000).
16. Vermes, I., Haanen, C., Steffens-Nakken, H. & Reutelingsperger, C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods.* **184**, 39-51 (1995).
17. Singh, N.P. Microgels for estimation of DNA strand breaks, DNA protein crosslinks and apoptosis. *Mutat. Res.* **455**, 111-127 (2000).
18. Konca, K. *et al.* A cross-platform public domain PC image-analysis program for the comet assay. *Mutat. Res.* **534**, 15-20 (2003).