Protection of the Flowers of *Prunus persica* Extract from Ultraviolet B-Induced Damage of Normal Human Keratinocytes

Young Ha Kim, Hye Eun Yang, Jong Ha Kim[1], Moon Young Heo, and Hyun Pyo Kim

College of Pharmacy, Kangwon National University, Chunchon 200-701 and
[1]R & D Center, Coreana Cosmetics Co. Ltd., Cheonan 333-830, Korea

(Received June 7, 2000)

For an attempt to develop safe materials protecting solar ultraviolet (UV)-induced skin damage, plant extracts were evaluated for their inhibitory activities of free radical generation and arachidonic acid/metabolites release from UVB-irradiated normal human keratinocytes. From the results of these screening procedures, the ethanol extract of the flowers of *Prunus persica* (Ku-35) was selected for further study. It was found that Ku-35 (100-1,000 μg/ml) inhibited the amount of ^{14}C-arachidonic acid/metabolites release from UVB-irradiated keratinocytes. It was also demonstrated that Ku-35 possessed the protective activity against UV-induced cytotoxicity of keratinocytes and fibroblasts. In addition, Ku-35 was revealed to protect UVB-induced erythema formation using guinea pigs in preliminary *in vivo* study. All these results indicate that the flowers of *P. persica* extract may be beneficial for protecting UV-induced skin damage when topically applied.

Key words: UV-induced skin damage, Flowers of *Prunus persica* (Rosaceae), Human keratinocyte, Arachidonic acid, Erythema

INTRODUCTION

Besides of many beneficial effects including vitamin D metabolism and melanin synthesis, solar ultraviolet (UV) radiation also gives notorious effects leading to skin erythema, skin aging including premature wrinkle formation, and ultimately skin carcinogenesis (Urbach, 1989; Streilein, 1995). Although the detailed cellular mechanisms are not fully understood, various investigations undertaken have shown that these processes comprise complex and multifaceted features. They include activation of signal transduction pathways involving protein kinases and transcriptional factors (Englaro *et al.*, 1998), release of cytokines, formation of arachidonic acid (AA)/metabolites, oxidative stress and ultimately DNA damage. To prevent these harmful effects, several kinds of UV blocking agents/sunscreens have been currently used in cosmetic industry, which absorb or scatter UV light not to reach into epidermal and dermal layers. However, there is a definite need of new materials to protect and/or prevent UV-induced photo-damage for safer and long-term uses. One example is an application of anti-oxidants such as vitamin C and/or E, which reduce oxidative damage caused by UV irradiation (Darr *et al.*, 1992; Tebbe *et al.*, 1997; Weber *et al.*, 1997), although there are always problems of stabilization of the final formulations. Other possible approach may be finding safe plant extracts to protect metabolic alterations of skin cells, especially epidermal layer, induced by UV irradiation. Since keratinocytes are the main cells in epidermal layer and one of the sites responsible for photo-damaging reactions, it is reasonable to think that materials protecting metabolic alteration of keratinocytes by UV exposure could have a beneficial effect on skin via preventing photo-damage. By UV irradiation, keratinocytes are activated and many cellular metabolisms are changed such as release of AA/metabolites from the membrane and formation of peroxidation products (Thiele *et al.*, 1998). Amount of released AA by UV irradiation was found to be related with formation of prostaglandin E_2 (PGE_2), one of chemical mediators of erythema, and closely associated with oxidative stress (Kang-Rotondo *et al.*, 1993; Chen *et al.*, 1996; Isoherranen *et al.*, 1999). For an attempt to develop plant materials protecting UV-induced skin damage, we have checked inhibitory potential of 200 plant extracts against free radical formation and UVB-induced AA/metabolites release from human keratinocyte culture. From the

Correspondence to: H. P. Kim, College of Pharmacy, Kangwon National University, Chunchon 200-701, Korea
E-mail: hpkim@cc.kangwon.ac.kr

results of these screening procedures, the ethanol extract of the flowers of *Prunus persica* (Ku-35) was selected for further study. This paper deals with the protective effects of Ku-35 against AA/metabolites release of human keratinocyte in culture. It is also shown that this extract strongly protected UVB-induced erythema of guinea pigs *in vivo*.

MATERIALS AND METHODS

Reagents and apparatus

[1-^{14}C]Arachidonic acid (AA, 54.6 mCi/mmol) was purchased from NEN (Boston, MA). Serum-free low calcium keratinocyte growth medium (KGM), DMEM, PBS, trypsin-EDTA, FBS and antibiotics were obtained from Gibco BRL (Grand Island, NY). Human recombinant epidermal growth factor (EGF) was purchased from Upstate Biotechnology (Lake Pacid, NY). Cholera toxin was a product of Difco Lab. (Detroit, MI). NIH/3T3 fibroblast cell line was obtained from American Type Culture Collection. Hydrocortisone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trypan blue and fatty acid-free bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). Silica gel 60 TLC plates were purchased from Merck (Germany). Other reagents used were chemicals of highest grade available.

Source of UV lamps

For irradiation to keratinocytes and fibroblasts, strength of UVB (Model XX-15B, medium wave length: 312 nm, Spectroline, Westbury, NY) and UVC (Model XX-15F, medium wave length:254 nm) was measured and adjusted using DRC-100X digital radiometer (Spectroline).

Plant materials

The flowers of *Prunus persica* (Rosaceae) were collected in several orchards located in Kangwon province (Korea) in April (1997 and 1998) and dried in the dark. A brochure specimen was deposited in College of Pharmacy (Kangwon National University). The plant material (1 kg) was extracted in 80% aqueous ethanol (10 L) at room temperature for 7 days. The extract was filtered and dried under vacuo giving 172 g of crude extract (Ku-35). The other plant materials such as *Uncaria sinensis,* Rubiaceae (thorn), *Rheum platum,* Polygonaceae (root), *Eugenia caryophyllata,* Myrtaceae (flower), *Curcuma longa,* Zingiberaceae (root) and *Alpinia officinale,* Zingiberaceae (root) were purchased from local market "Kyung-Dong" (Seoul, Korea). These plant materials were extracted and dried using the same procedures described above. For cell culture study, the plant extracts were dissolved in PBS. For *in vivo* study, Ku-35 was emulsified in oil-based vehicle.

Cell cultures

Normal human keratinocytes were cultured essentially according to the established method of Boyce and Ham (1983) using serum-free keratinocyte growth medium (KGM). In brief, human foreskin was obtained and subcutaneous fat tissues were trimmed off. The tissue was cut into approximately 0.5×1.0 cm^2 size and incubated in 0.25% trypsin-EDTA at 4°C overnight. The obtained keratinocytes were initially suspended in DMEM with 10% FBS, 1% glutamine, 1% antibiotics, 10 ng/ml EGF, cholera toxin and 4 ug/ml hydrocortisone. After cells were counted and viability was checked using MTT assay, keratinocytes were plated at 1.0×10^6 cells/100 mm plate and incubated at 37°C in 5% CO_2. Two days after initial seeding, media was changed to KGM supplemented with EGF and pituitary extract. After reaching 70-80% confluent, cells were detached by trypsin-EDTA treatment and cultured in the same media. In order to check cell viability, MTT solution (5 mg/ml) was added to each well and incubated for 4 h. The formazan crystals were dissolved by addition of DMSO (200 μl) and measured at 570 nm as previously described by Mosmann (1983). Protein concentration was measured with Bio-Rad reagent according to the manufacturers manual and DNA was quantitated with ethidium bromide fluorescence technique. For arachidonic acid labeling and release study, keratinocytes within fourth passage were always used.

Arachidonic acid (AA) labeling and release assay

Keratinocytes were plated in 24-well plates (2×10^4 cells/well). When cultures were 80-90% confluent, cells were labeled with 0.0375 uCi/ml [^{14}C]AA in KGM without supplements for 24 h. The media was removed and washed 3 times with KGM containing 1 mg/ml fatty acid-free BSA. For checking distribution pattern of ^{14}C-AA in the membrane, cells were extracted with chloroform:methanol (2:1) and organic phase was dried under N_2. Lipids were analyzed using TLC with chloroform:methanol:acetic acid:water (90:8:1:0.8) as a mobile phase, in which glycerophospholipids remain on original spotting area. For examining the inhibitory effects of the plant extracts, cells were pre-incubated with extracts for 1 h. Immediately before irradiation, the medium was replaced by small amount of calcium/magnesium-free PBS to avoid drying during UVB irradiation. The PBS contained the extract in the same concentration as in the pre-incubation, while control group only received same amount of PBS. After UVB (30 mJ/cm^2) was irradiated, KGM containing the same amount of the extract was re-added and cells were further incubated for 6 h. The medium was removed and washed twice with KGM containing 1 mg/ml fatty acid-free BSA. The combined medium was centrifuged to remove cell debris. Radioactivity of small amount of the supernatant was measured in toluene-based scintillant and considered as total release (^{14}C-AA/^{14}C-containing

other cellular metabolites). For measuring ^{14}C-AA release, the remaining supernatant was extracted with chloroform: methanol (2:1). The organic layer was dried under N_2 and ^{14}C-AA was separated with TLC using petroleum ether:diethyl ether:acetic acid (50:50:1) as a mobile phase according to the procedure of Kim *et al.* (1998). ^{14}C-AA released co-migrating with authentic standard was scraped out after visualization with iodine vapor and radioactivity was counted. In some cases, autoradiography was carried out for 7 days to find radioactive spots on TLC plates.

Protection of UV-induced cytotoxicity of fibroblast

NIH/3T3 fibroblast cell line was cultured in DMEM with 10% FBS, 2% glutamine (200 mM) and 2% penicillin-streptomycin (10,000 U/ml) using 96-well plates (2.5×10^4 cells/well). For pre-treatment study, Ku-35 was added at 0-500 ug/ml and incubated for 24 h. After the medium was removed and small amount of PBS was added, UVB (50 mJ/cm^2) or UVC (5 mJ/cm^2) was irradiated. Fresh medium was added and incubated further for 2 h. In post-treatment experiment, the medium containing Ku-35 was added after UV irradiation. Subsequently, the cells were cultured for 14 h. Cell viability was accessed with MTT reduction assay as described above.

In vivo erythema test

Male SPF Hartly guinea pigs (250-300 g) were obtained from Korea Experimental Animal Center and acclimatized in our animal facility (KNU) at least for 7 days prior to experiment. Dorsal hair of animals was cut and depilated with application of Nair®. Twenty-four hours later, Ku-35 emulsified in oil-based vehicle was applied, while control group received vehicle only. After UVB (2 J/cm^2) was irradiated to lightly anethesized animal with diethyl ether, erythema formation was visually observed in various time intervals.

RESULTS AND DISCUSSION

By exposure to UV light, skin experiences the dynamic changes of metabolism including formation of eicosanoids and peroxidation products. Epidermis is one of the sites for this change, in which keratinocytes are major cells. Thus, activation of keratinocytes by UV irradiation is an important factor for skin erythema, premature aging and skin carcinogenesis. For finding the materials to prevent these processes, the effects on AA/metabolites release from UVB-activated keratinocytes were measured using 200 plant extracts, in addition to their inhibitory activity of free radical formation. From these screening procedures, the flowers of *P. persica* extract was found to be one of the promising materials for protecting UV-induced skin damage and studied further in this investigation.

When normal human keratinocytes were cultured, their initial viability was more than 90%. ^{14}C-AA (0.0375 uCi/ml) was incorporated to keratinocytes for 24 h at 95.7 ± 3.2% yield (n=4). Using extraction and TLC separation techniques, it was revealed that the labeled AA was incorporated mainly to polar lipid class, glycerophospholipid (data not shown). These results were similar with the previous findings of Kang-Rotondo *et al.* (1993). Under this standard condition, plant extracts were added and UVB was irradiated. The radioactivity released to medium for 6 h period was counted and considered as total release (^{14}C-AA/^{14}C-containing other cellular metabolites). In order to determine the irradiation strength of UVB, various amount of UVB (1-100 mJ/cm^2) were irradiated. From irradiation strength of 30 mJ/cm^2, total release was sharply increased, but the significant cytotoxicity (more than 50%) was observed over 50 mJ/cm^2 (data not shown). Therefore, UVB (30 mJ/cm^2) was used throughout this study. At this strength, about 1.7-2.5 fold increase of total release from keratinocytes was observed compared to the amount of sham irradiated group depending on the experiments. The effects of several plant extracts on AA/metabolites release were shown in Fig. 1. The plant extracts such as *U. sinensis*, *P. persica* and *C. longa* clearly possessed the inhibitory activity against UVB-induced total release from keratinocytes. Because the flowers of *P. persica* extract (Ku-35) showed inhibitory activity of free radical generation in preliminary screening procedure and UVB-induced release of AA/metabolites (Fig. 1), Ku-35 was selected for further study. When Ku-35 was added and ^{14}C-AA released was analyzed using TLC separation and autoradiographic techniques, the amounts of ^{14}C-AA as well as total radioactive materials

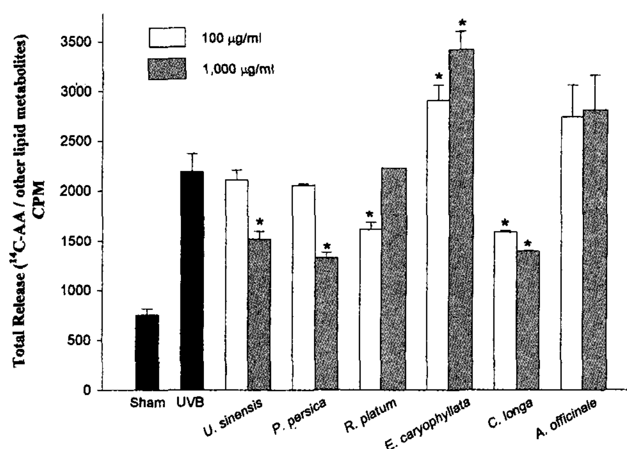

Fig. 1. Effects of several plant extracts on ^{14}C-AA/metabolites release from UVB irradiated keratinocytes. Total radioactivity release from keratinocytes was counted under standard condition for 6 h incubation period. Data were represented as arithmetic mean ± SD (n=3) and statistical significance was evaluated with Students t-test., *: $P<0.005$, Significantly different from UVB irradiated group.

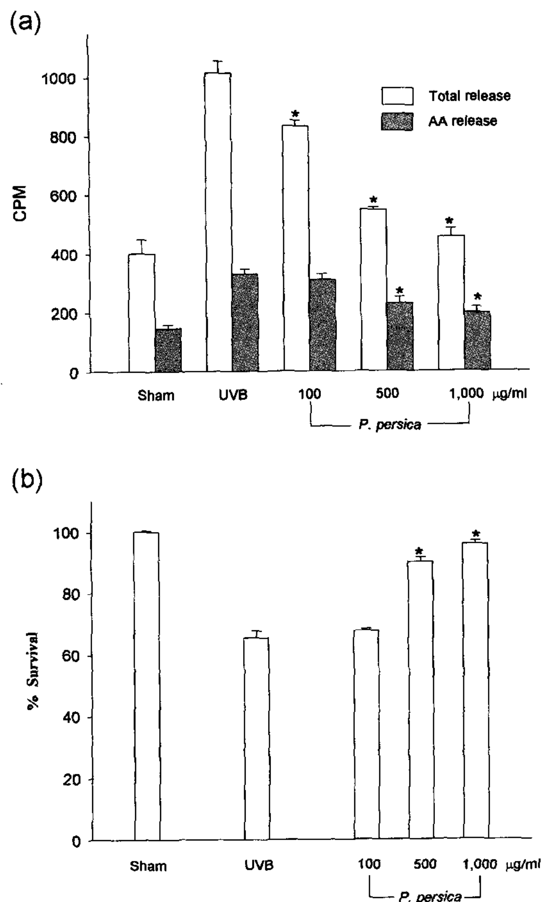

Fig.. 2. Concentration-dependent effects of the flowers of *P. persica* extract (Ku-35) on ^{14}C-AA release and cytotoxicity of keratinocytes. (a)Effects on ^{14}C-AA and total release from UVB irradiated keratinocytes., For measuring ^{14}C-AA release, the culture medium treated with UVB and Ku-35 was extracted with chloroform:methanol (2:1) and the organic layer was dried. The reaction products were separated with TLC and autoradiographed as described in materials and methods section. ^{14}C-AA spots were scraped and the radioactivity was counted., (b) Effects on cytotoxicity of keratinocytes., MTT reduction assay was used for measuring cytotoxicity of keratinocytes., Data were represented as arithmetic mean ± SD (n=3) and statistical significance was evaluated with Students t-test., *:P< 0.005, Significantly different from UVB irradiated group.

released were significantly reduced in a concentration-dependent manner at 100-1,000 µg/ml (Fig. 2a). The IC_{50} values of Ku-35 were found to be approximately 429 and 207 ug/ml for ^{14}C-AA and total release, respectively. When protective activity of Ku-35 against UVB-induced cytotoxicity was measured using MTT bioassay, UVB irradiation (30 mJ/cm^2) decreased viability of keratinocytes (31 ± 4%) while Ku-35 (100-1000 µg/ml) significantly reversed UVB-induced cytotoxicity of keratinocytes (Fig. 2b). These findings strongly suggest that Ku-35 may protect keratinocyte activation and epidermal damage by UVB irradiation. In order to examine effects of Ku-35 on skin fibroblasts, major cells of dermal layer, UVB or C was irradiated to NIH/3T3 cells, followed by measuring cytotoxicity. Table I demonstrated the protective effects of Ku-35. Against UVB irradiation, Ku-35 reduced cytotoxicity, although not statistically significant. In addition, Ku-35 significantly reversed UVC-induced cytotoxic effect at 100 ug/ml in pre-treatment and 500 ug/ml in post-treatment. These results strongly suggest that Ku-35 may give favorable effects for skin protection against UV irradiation when topically applied. This speculation is also supported by the fact that Ku-35 almost completely inhibited UVB-

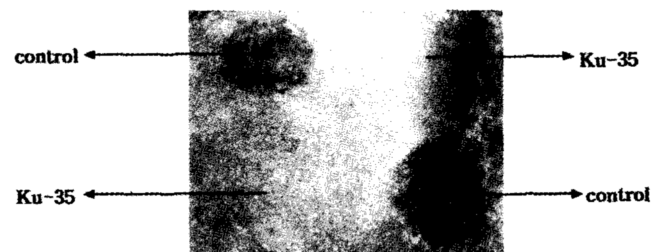

Fig. 3. Protection of the flowers of *P. persica* extract (Ku-35) from UVB-induced erythema. Ku-35 (10 mg/cm^2) was applied and UVB was irradiated on the depilated skin of guinea pigs as described in experimental section., The photograph here was taken 6 day after UVB irradiation, showing no signs of erythema and edema in Ku-35-treated group whereas significant erythema was noted in control area., The protective activity of Ku-35 against erythema formation was observed as early as 3 h after UVB irradiation.

Table I. Effects of the flowers of *P. persica* extract (Ku-35) on cytotoxicity of skin fibroblast, NIH/3T3

Concentration (ug/ml)	% Cell viability			
	UVB		UVC	
	Pre-[a]	Post-treatment[a]	Pre-[a]	Post-treatment[a]
0	97 ± 13[b]	39 ± 17	79 ± 26	57 ± 6
50[c]	94 ± 21	35 ± 10	86 ± 21	59 ± 14
100	105 ± 12	34 ± 7	100 ± 8*	61 ± 15
250	106 ± 23	35 ± 7	69 ± 26	64 ± 12
500	100 ± 11	41 ± 8	60 ± 21	79 ± 20**

[a]Ku-35 was treated as described in experimental section., [b]All values here were represented as arithmetic mean ± SD by MTT assay (n=6), compared to sham irradiated group., [c]Ku-35 alone (0-500 ug/ml) did not show any cytotoxicity., *: P< 0.1, **: P< 0.05, Significantly different from UV irradiated group without Ku-35.

induced erythema and edema formation at a dose of 10 mg/cm^2 in preliminary *in vivo* study (Fig. 3). Since amount of AA released is known to be parallel with formation of PGE_2, one of major mediators of erythema, the protective potential of Ku-35 against UVB-induced erythema may be mediated, at least in part, by preventing AA release, thereby reducing PGE_2 formation. All results from this investigation indicate that Ku-35 clearly possesses protective activity against UV-induced skin damage judged by inhibitions of AA/metabolites release, cytotoxicity of keratinocytes and skin fibroblasts, and *in vivo* erythema formation. The investigation to elucidate the detailed action mechanism of UV protection is now under progress.

From our study, it is concluded that the flowers of *P. persica* extract may have a potential for protecting UV-induced skin damage. It could be used as a new cosmetic ingredient being special for UV protection.

ACKNOWLEDGEMENTS

This study was financially supported by research grant from Ministry of Health and Welfare, Korea (HMP-97-D-5-0034) and greatly acknowledged.

REFERENCES

Boyce, S. T. and Ham, R. G., Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum free serial culture. *J. Invest. Dermatol.*, 81 (Suppl.), 33S-40S (1983).

Chen, X., Gresham, A., Morrison, A. and Pentland, A. P., Oxidative stress mediates synthesis of phospholipase A_2 after UVB injury. *Biochim. Biophys. Acta,* 1299, 23-33 (1996).

Darr, D., Combs, S., Dunston, S., Manning, T. and Pinnell, S., Topical vitamin C protects porcine skin from ultraviolet-induced damage. *Br. J. Dermatol.*, 127, 247-253 (1992).

Englaro, W., Derijard, B., Ortonne, J-P. and Ballotti, R., Solar ultraviolet light activates extracellular signal-regulated kinases and the ternary complex factor in human normal keratinocytes. *Oncogene,* 16, 661-664 (1998).

Isoherranen, K., Punnonen, K., Jansen, C. and Uotila, P., Ultraviolet irradiation induces cyclooxygenase-2 expression in keratinocytes. *Br. J. Dermatol.*, 140, 1017-1022 (1999).

Kang-Rotondo, C. H., Miller, C. C., Morrison, A. R. and Pentland, A. P., Enhanced keratinocyte prostaglandin synthesis after UV injury is due to increased phospholipase activity. *Am. J. Physiol.*, 264, C396-C401 (1993).

Kim, H. P., Indu, M., Iversen, L. and Ziboh, V. A., Effects of naturally-occurring flavonoids and biflavonoids on epidermal cyclooxygenase and lipoxygenase from guinea-pigs. *Prostg. Leukot. Essen. Fatty Acids,* 58, 17-24 (1998).

Mosmann, T., Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxic assays. *J. Immunol. Methods,* 65, 55-63 (1983).

Streilein, J. W., UVB susceptibility: Possible relationship to photoaging and Photocarcinogenesis, In Gilchrest, B. A. (Ed.). *Photoaging*. Cambridge, New York, pp. 68-80, 1995.

Tebbe, B., Wu, S., Geilen, C. C., Erberle, J., Kodelja, V. and Orfanos, C. E., L-Ascorbic acid inhibits UVA-induced lipid peroxidation and secretion of IL-1a and IL-6 in cultured human keratinocytes *in vitro*. *J. Invest. Dermatol.,* 108, 302-306 (1997).

Thiele, J, J., Traber, M. G. and Packer, L., Depletion of human stratum corneum vitamin E: an early and sensitive *in vivo* marker of UV induced photo-oxidation. *J. Invest. Dermatol.*, 110, 756-761 (1998).

Urbach, F., Potential effects of altered solar ultraviolet radiation on human skin cancer. *Photochem. Photobiol.,* 50, 507-513 (1989).

Weber, C., Podda, M., Rallis, M., Thiele, J. J., Traber, M. G. and Packer, L. Efficacy of topically applied tocopherols and tocotrienols in protection of murine skin from oxidative damage induced by UV-irradiation. *Free Rad. Biol. Med.*, 22, 761-769 (1997).