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European Journal of Pharmacology 476 (2003) 173–178
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Inhibition of ultraviolet-A-modulated signaling pathways by asiatic acid and ursolic acid in HaCaT human keratinocytes

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Received 10 March 2003; received in revised form 22 July 2003; accepted 29 July 2003

Abstract

Premature aging of the skin (photoaging) is a well-documented consequence of exposure to ultraviolet-A (UVA). Enhanced generation of reactive oxygen species and induction of matrix metalloproteinases (MMPs) appear to be the most important components of UVA-modulated signal transduction pathways, ultimately leading to photoaging. In this study, we investigated the effects of asiatic acid and ursolic acid, triterpene compounds, on the UVA-modulated signaling pathways using HaCaT human keratinocytes as a model cellular system. In the cells, we confirmed that UVA irradiation induced oxidative stress and increased the expression of MMP-2. Asiatic acid and ursolic acid significantly suppressed the UVA-induced reactive oxygen species production and lipid peroxidation. Pretreatment with asiatic acid or ursolic acid significantly reduced the UVA-induced activation and expression of MMP-2. In addition, UVA-induced enhanced expression of p53, a hallmark of UV-induced DNA damage and cell death, was also significantly inhibited by pretreatment with asiatic acid or ursolic acid. Taken together, these results suggest that asiatic acid and ursolic acid may be an effective inhibitor of UVA-modulated signal transduction pathways in human skin cells. These results further suggest that these agents may be useful in the prevention of UVA-induced photoaging.

Keywords: Asiatic acid; Ursolic acid; HaCaT human keratinocyte; Reactive oxygen species; Matrix metalloproteinase; p53

1. Introduction

The skin is the most susceptible organ to damage by ultraviolet (UV) irradiation as it is directly exposed to UV light. Solar UV irradiation is divided into three components, UVA (320–400 nm), UVB (280–320 nm) and UVC (200–280 nm). Since the UVC component is blocked by the earth's ozone layer, UVA and UVB are responsible for numerous biological effects in the skin, including premature aging characterized by wrinkles, leathery texture and mottled pigmentation (Gilchrest and Yaar, 1992; Scharffetter-Kochanek et al., 1997). Molecular mechanisms of skin wrinkles are probably due to the loss of macromolecules making up the dermal matrix (Gilchrest, 1996; West, 1994), among which collagen is the major component. The level of collagen in normal skin is maintained by the balance between synthesis by dermal fibroblasts and enzymatic degradation. In UV-irradiated skin, the level of matrix metalloproteinases (MMPs) that are important enzymes for the proteolysis of extracellular matrix proteins is elevated long before the visible symptoms of photoaging (Fisher et al., 1996, 1997). Among them, MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa), secreted as proenzymes, play an important role in degrading type IV collagen (Johnson et al., 1998; Kobayashi et al., 1998). Inhibition of induction of MMPs has been reported to alleviate UV-induced photoaging by preventing from collagen destruction (Fisher et al., 1996; Oikarinen et al., 1993).

Reactive oxygen species are generated in UVA-irradiated human skin, resulting in oxidative damage to lipids, proteins, and DNA (Cunningham et al., 1985; Hanson and Clegg, 2002; Vile and Tyrrell, 1995). UV-radiated DNA damage further activates p53, leading to the apoptosis of keratinocytes, in turn, disruption of the epithelial structure (Qin et al., 2002). Reactive oxygen species produced by UV

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doi:10.1016/S0014-2999(03)02177-0

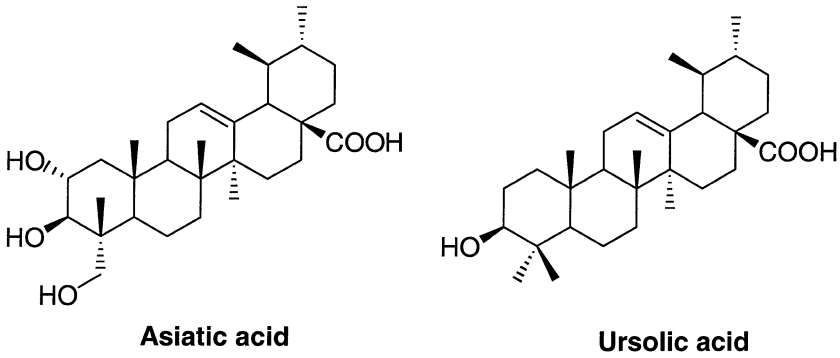

Fig. 1. Chemical structure of asiatic acid and ursolic acid.

irradiation regulate gene expression, including induction of MMPs (Berneburg et al., 1999; Scharffetter-Kochanek et al., 2000; Wenk et al., 2001).

Asiatic acid, a pentacyclic triterpene compound found in *Centella asiatica*, has been traditionally used as a tonic in skin diseases and leprosy (Shukla et al., 1999). Ursolic acid, structurally very similar to asiatic acid as shown in Fig. 1, widely exists in all parts of a variety of plants and has been reported to possess a variety of biological effects (Mahato et al., 1988). In particular, ursolic acid has been known to increase collagen content in human skin in addition to other actions such as anti-inflammatory, skin-tumor prevention (Huang et al., 1994) and anti-invasion (Cha et al., 1996). Asiatic acid has been shown to promote fibroblast proliferation and collagen synthesis and to stimulate extracellular matrix accumulation in a rat wound model (Maquart et al., 1999). However, to our knowledge, effects of asiatic acid and ursolic acid on UV irradiation-modulated reactive oxygen species production and induction of MMPs have not been reported yet.

Thus, in the present study, we investigated the effects of asiatic acid and ursolic acid on the UVA-modulated signaling pathways using HaCaT human keratinocytes as a model cellular system. In particular, we focused their effects upon reactive oxygen species production, induction of MMPs and p53 expression. We also tried to compare their effects with those of retinoic acid, a well-known anti-wrinkle agent (Fisher et al., 1999).

2. Materials and methods

2.1. Cell culture

The HaCaT human keratinocyte cell line was purchased from ATCC. HaCaT cells were grown at 37 °C in a humidified incubator under 5% CO_2/95% air in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 200 IU/ml penicillin, 200 mg/ml of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every other day. After confluence, the cells were subcultured following trypsinization.

2.2. UVA irradiation

Cells were irradiated using a UV radiator (Lilber Lourmat, France) emitting wavelength 365 nm for 1–4 min, of which dose were set at 80–320 mJ/cm^2. Following irradiation, the cells were incubated for further 12 h.

2.3. Cell viability assay (MTT staining)

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) staining method (Van de Loosdrecht et al., 1991). Cells from 4- to 5-day-old cultures were seeded in 24-well plates at the density of 5×10^4 cells/well. The volume of the medium in the wells was 1 ml. In control experiments, cells were grown in the same media containing drug-free vehicle. After incubation with drug for 48 h, 100 μl of MTT (5 g MTT/l in H_2O) was added and cells incubated for a further 4 h. Two hundred microliters of DMSO was added to each culture and mixed by pipetting to dissolve the reduced MTT crystals. Relative cell viability was obtained by scanning with an enzyme-linked immuno sandwich assay (ELISA) reader (Molecular Devices, Menlo Park, CA) with a 540-nm filter.

2.4. Cytochrome c reduction assay

The level of cytochrome *c* reduction was measured to assess superoxide production in the cells. Cell medium was changed to fresh medium without fetal bovine serum and treated with drugs or UV. After treatment, the conditioned media of 1×10^5 cells were mixed with 50 μM cytochrome *c* and incubated at 37 °C for 15 min. Another set of the same mixtures was reserved at 0 °C for use as a blank. Incubation was terminated by placing the mixtures at 0 °C. After centrifugation of blanks and incubated mixtures at 30,000 g for 5 min at 4 °C, the absorbance of the incubated supernatant was read spectrophotometrically at 550 nm using unincubated blank as a reference.

2.5. Lipid peroxidation assay

The cells harvested were suspended in 1 ml of PBS and mixed with 0.2 ml of 8.1% sodium dodecylsulfate (SDS), 1.5 ml of 20% acetic acid solution (pH 3.5), and 1.5 ml of 0.8% thiobarbituric acid. The mixture was heated at 95 °C for 1 h, chilled to room temperature, and extracted with 1 ml of ddH_2O and 2.5 ml of *n*-butanol·pyridine mixture (15:1, v/v). The upper organic layer containing malondialdehyde produced by lipid peroxidation was measured at 532 nm. Synthetic malondialdehyde was used as an external standard, and the level of lipid peroxides was expressed as nM of malondialdehyde. The protein concentration was measured by the method of Bradford (Denis et al., 2000).

2.6. Zymography

Enzyme activity of MMP-2 was assayed by gelatin zymography according to the method of Herron et al. (1986). The conditioned media of 1×10^4 cells were separated by electrophoresis on a 10% SDS-polyacrylamide gel containing 0.33 mg/ml gelatin. The electrophoresed gel was washed twice with washing buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 2.5% triton X-100, followed by a brief rinsing in washing buffer without triton X-100. Then, the gel was incubated with incubation buffer of 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3 1 μM $ZnCl_2$ at 37 °C. After incubation, the gel was stained with 0.25% Coomassie blue R250 and destained with 7% acetic acid. Presence of the MMP was identified with a clear zone of gelatin digestion. The density of bands for proMMP-2 and MMP-2 was measured by using Image analyzing system (UVP, Upland, USA).

2.7. Western blot analysis

Cells were washed with PBS solution and centrifuged at 1000×*g* for 5 min. Cell pellets were lysed for 15 min at 4 °C in whole cell extraction buffer containing 50 mM HEPES (pH 7.4), 0.5% Nonidet P-40, 10% glycerol, 137 mM NaCl, 1 mM EGTA, 10 mM NaF, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 40 mM α-glycerophosphate, 0.1 mM dithiothreitol. Lysates were centrifuged at 20,000×*g* for 10 min at 4 °C, and the concentration of the supernatant proteins was determined by using Bradford protein assay kit (Sigma, St. Louis, MO, USA). Equal amount of the proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to Hybond ECL nitrocellulose membrane (Amersham Life Science, Buckinghamshire, England) at 30 V for overnight. The membrane was blocked with 5% skim milk in Tween-20 containing Tris-buffered saline (TTBS) (20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20) and incubated with primary anti-human MMP-2 (Oncogene Research Products, Boston, USA) with dilution of 1:2000 or p53 (Santa Cruz Biotechnology, California, USA) antibody with dilution of 1:3000 in TTBS containing 5% skim milk. After incubation with horseradish peroxidase-conjugated anti-immunoglobulin G antibody (Santa Cruz Biotechnology) with dilution of 1:2000, immunodetected proteins were visualized by using enhanced chemiluminescence assay kit (Amersham Life Science). Equal loading of the samples was assured by the staining of protein-transferred membrane with ponceau S. The density of bands for proMMP-2 and MMP-2 were measured by using Image analyzing system (UVP).

2.8. Materials

The powders for MEM, trypsin solution, MTT, sodium pyruvate, and all salt powders were obtained from Sigma. Fetal bovine serum and antibiotics were purchased from GIBCO (Grand Island, NY, USA). The stock solutions of drugs were sterilized by filtration through 0.2-μm disc filters (Gelman Sciences: Ann Arbor, MI, USA).

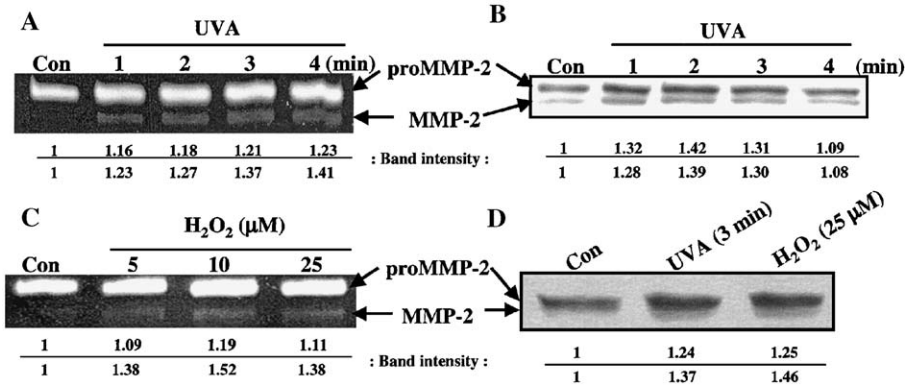

Fig. 2. Enhancement of the activation and expression of MMP-2 by UVA irradiation and H_2O_2 in HaCaT human keratinocytes. Cells were supplemented with PBS 24 h prior to UVA (365 nm) irradiation or H_2O_2 treatment, and harvested for 12 h after the treatment. Gelatin zymography (A, C) and western blot analysis (B, D) were performed.

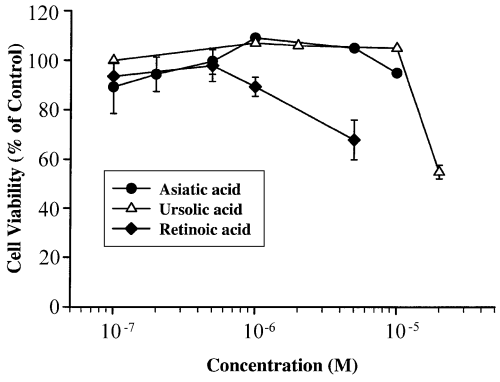

Fig. 3. The effects of asiatic acid, ursolic acid and retinoic acid on the viability of HaCaT cells. Cells treated for 24 h with or without each concentration of drugs were analyzed for viability by MTT assay.

2.9. Data analysis

All experiments were performed four times. Data were expressed as mean±standard error of the mean (S.E.M.) and were analyzed using one-way analysis of variance (ANOVA) and Student–Newman–Keul's test for individual comparisons. *P* values less than 0.05 are considered statistically significant.

3. Results

3.1. Induction of MMP-2 by UVA irradiation

The effect of UVA (365 nm) irradiation on the gelatinolytic activity of MMP-2 was examined using the zymographic analysis in HaCaT human keratinocytes. Irradiation of UVA 1 to 4 min corresponding to 80–320 mJ/cm^2 of UVA increased proMMP-2 activation in a dose-dependent manner as shown in Fig. 2A. UVA irradiation also increased the protein expression of MMP-2 up to 3 min-exposure, but 4 min-exposure of UVA did not increase the level of MMP-2 in the cells as shown in Fig. 2B. Since ROS appear to play an important role in induction of MMPs by UV irradiation, we investigated whether exogenous application of H_2O_2 can mimic the effects of UVA irradiation on the activation and induction of MMP-2. As shown in Fig. 2C, H_2O_2 activated proMMP-2 in a concentration-dependent manner. Protein activity of MMP-2 was also induced by H_2O_2 in a dose-related manner (Fig. 2C) similar to the effect of UVA irradiation (Fig. 2D). These results suggest that ROS may mediate UVA irradiation-modulated induction of MMP-2.

3.2. Inhibition of activation and induction of MMP-2 by asiatic acid and ursolic acid

The effects of ursolic acid and asiatic acid, structurally similar triterpene compounds (Fig. 1), on the UVA irradiation-modulated activation and induction of MMP-2 were

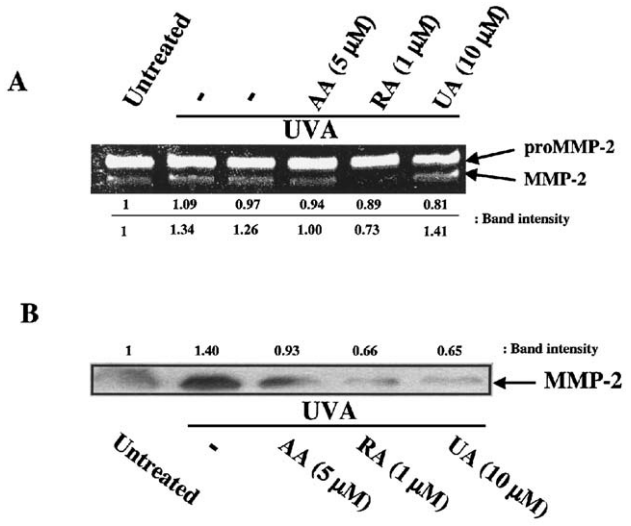

Fig. 4. Inhibition of the UVA-induced activation and induction of MMP-2 by asiatic acid (AA), ursolic acid (UA) and retinoic acid (RA) in HaCaT human keratinocytes. The cells were incubated with AA, UA or RA for 24 h prior to UVA (365 nm) irradiation for 3 min. In the experiments' activities (A) and expression (B) of MMP-2 were measured by gelatin zymograpy and western blot, respectively.

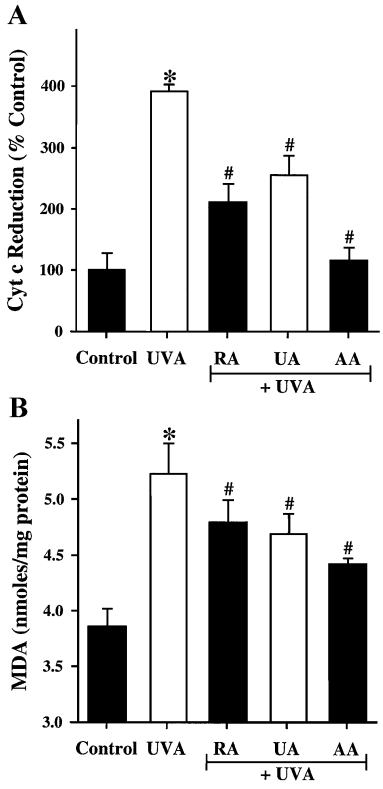

Fig. 5. Asiatic acid (AA), ursolic acid (UA) and retinoic acid (RA) suppress the basal generation of ROS (A) and lipid peroxidation (B) in HaCaT human keratinocytes. Cells were treated for 24 h with or without drugs prior to UVA (365 nm, 3 min) irradiation. In the experiments AA (5 μM), UA (10 μM) and RA (1 μM) were used. Data points represent the mean values of four replications with bars indicating S.E.M. *P<0.05 compared to control. $^{\#}P$<0.05 compared to UVA-irradiated group.

investigated. The maximum concentrations of asiatic acid and ursolic acid without affecting cell viability were determined using the MTT assay before examining their effects on the expression of MMPs in HaCaT cells. The data (Fig. 3) show that maximum concentrations without cytotoxic effects were 5 and 10 μM for asiatic acid and ursolic acid, respectively, which were higher than that for retinoic acid (1 μM), a well-known anti-wrinkle agent. Pretreatment with asiatic acid (5 μM) or ursolic acid (10 μM) did not affect UVA irradiation-modulated activity of proMMP-2. However, asiatic acid (5 μM) or ursolic acid (10 μM) inhibited UVA-induced activity of MMP-2, which although seems to be less effective than the effect of retinoic acid (1 μM) as depicted in Fig. 4A. Furthermore, the expression level of MMP-2 by UVA irradiation was significantly suppressed by pretreatment with asiatic acid (5 μM) or ursolic acid (10 μM) (Fig. 4B).

3.3. Inhibition of UVA irradiation-induced reactive oxygen species generation and lipid peroxidation by asiatic acid and ursolic acid

The effects of asiatic acid and ursolic acid on UVA irradiation-induced reactive oxygen species generation and lipid peroxidation in HaCaT cells were also investigated using cytochrome *c* reduction assay and lipid peroxidation assay, respectively. As shown in Fig. 5A, UVA irradiation significantly increased reactive oxygen species production and lipid peroxidation in the cells, which was significantly prevented by asiatic acid (5 μM), ursolic acid (10 μM) and retinoic acid (1 μM) as shown in Fig. 5B.

3.4. Inhibitory effects of asiatic acid and ursolic acid on UVA irradiation-induced p53 expression

Since apoptosis of keratinocytes leading to disruption of the epithelium in UV-irradiated skin seems to be related to the activation of p53, the effects of asiatic acid and ursolic acid on the UVA-induced expression of p53 were examined.

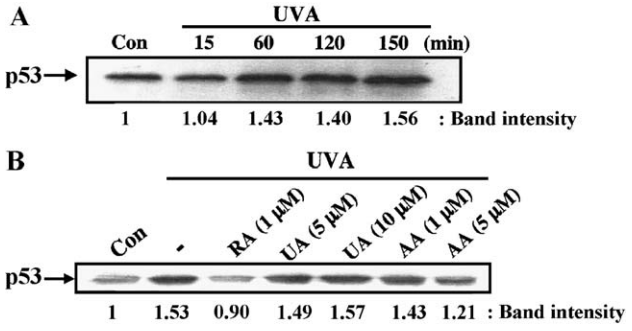

Fig. 6. Inhibition of the UVA irradiation-induced enhanced expression of p53 by the pretreatment with asiatic acid (AA), ursolic acid (UA) and retinoic acid (RA) in HaCaT human keratinocytes. Cells alone or pretreated with AA, UV or RA for 24 h were irradiated with UVA (365 nm) for 3 min, and further harvested for a designated time (A) or for 150 min (B), then analyzed for Western blotting.

As shown in Fig. 6, p53 was highly expressed in the UVA-irradiated HaCaT cells, which was significantly reduced by pretreatment with 1 and 5 μM of asiatic acid, 5 and 10 μM of ursolic acid and 1 μM of RA. The response to retinoic acid was the most effective. The inhibitory effect of asiatic acid on the UVA-induced p53 increase was dose-dependent, while that of ursolic acid was not the case. Although speculated, inhibitory effects of asiatic acid on p53 activation may be beneficial to reduce UVA irradiation-dependent skin cell killing, and in turn, photoaging.

4. Discussion

The studies on development of novel agents with anti-photoaging capabilities particularly from natural resources including various plants have been intensively performed. Since inhibition of MMPs appears to be useful intervention for skin wrinkle (Fisher et al., 1999), agents inhibiting induction of MMPs are screened for preventing photoaging. The major findings of this study are that UVA irradiation-modulated signaling pathways (e.g., reactive oxygen species production, induction of MMP-2 and accumulation of apoptosis-related p53) were effectively blocked by asiatic acid and ursolic acid, triterpene compounds, in human keratinocytes.

Previously, asiatic acid and other triterpenes from *C. asiatica* have been reported to show wound healing activity through stimulation of collagen synthesis in various model systems (Bonte et al., 1994; Maquart et al., 1990). Ursolic acid has also been shown to increase the ceramide and collagen contents in human skin cells (Both et al., 2002; Yarosh et al., 2000). Since molecular mechanism of skin wrinkles seems to be due to the loss of collagen component of the dermal matrix by enhanced enzymatic degradation through the induction of MMPs (Gilchrest, 1996; West, 1994), in addition to stimulation of collagen synthesis, inhibitory effects of asiatic acid and ursolic acid on induction of MMP-2 as shown in this study (Fig. 4) strongly suggest that asiatic acid and ursolic acid may be valuable for protection from UV-mediated skin wrinkle.

Reactive oxygen species have been generated by UV irradiation in cultured human skin fibroblasts (Masaki et al., 1995; Vile and Tyrrell, 1995). Reactive oxygen species appear to play a central role in UV irradiation-mediated apoptotic cell death (Kulms et al., 2002), and increased accumulation of p53, an essential factor for apoptosis triggered by UV irradiation (Vile, 1997). The present study also clearly demonstrated that UVA irradiation induced reactive oxygen species production and lipid peroxidation (Fig. 5) and p53 accumulation in human keratinocytes (Fig. 6), and that asiatic acid and ursolic acid significantly suppressed the UVA irradiation-induced reactive oxygen species production, lipid peroxidation and p53 accumulation (Figs. 5 and 6).

In conclusion, asiatic acid and ursolic acid, triterpene compounds, prevented UVA irradiation-induced reactive oxygen species production, lipid peroxidation and induction of MMP-2 as well as p53 in HaCaT human keratinocytes. These results suggest that asiatic acid and ursolic acid may be an effective inhibitor of UVA-modulated signal transduction pathways in human skin cells. These results further suggest that these agents may be valuable in the prevention of UVA-induced photoaging.

Acknowledgements

This work was supported by Korea Food and Drug Administration Research Grant 2001.

References

Berneburg, M., Grether-Beck, S., Kurten, V., Ruzicka, T., Briviba, K., Sies, H., Krutmann, J., 1999. Singlet oxygen mediates the UVA-induced generation of the photoaging-associated mitochondrial common deletion. J. Biol. Chem. 274, 15345–15349.

Bonte, F., Dumas, M., Chaudagne, C., Meybeck, A., 1994. Influence of asiatic acid, madecassic acid, and asiaticoside on human collagen I synthesis. Planta Med. 60, 133–135.

Both, D.M., Goodtzova, K., Yarosh, D.B., Brown, D.A., 2002. Liposome-encapsulated ursolic acid increases ceramides and collagen in human skin cells. Arch. Dermatol. Res. 293, 569–575.

Cha, H.J., Bae, S.K., Lee, H.Y., Lee, O.H., Sato, H., Seiki, M., Park, B.C., Kim, K.W., 1996. Anti-invasive activity of ursolic acid correlates with the reduced expression of matrix metalloproteinase-9 (MMP-9) in HT1080 human fibrosarcoma cells. Cancer Res. 56, 2281–2284.

Cunningham, M.L., Krinsky, N.I., Giovanazzi, S.M., Peak, M.J., 1985. Superoxide anion is generated from cellular metabolites by solar radiation and its components. J. Free Radic. Biol. Med. 1, 381–385.

Denis, G., Humblet, C., Verlaet, M., Boniver, J., Defresne, M.P., 2000. p53, Bax and Bcl-2 in vivo expression in the murine thymus after apoptogenic treatments. Anticancer Res. 18, 3315–3321.

Fisher, G.J., Datta, S.C., Talwar, H.S., Wang, Z.Q., Varani, J., Kang, S., Voorhees, J.J., 1996. Molecular basis of sun-induced premature skin ageing and retinoid antagonism. Nature 379, 335–339.

Fisher, G.J., Wang, Z.Q., Datta, S.C., Varani, J., Kang, S., Voorhees, J.J., 1997. Pathophysiology of premature skin aging induced by ultraviolet light. N. Engl. J. Med. 337, 1419–1428.

Fisher, G.J., Talwar, H.S., Lin, J., Voorhees, J.J., 1999. Molecular mechanisms of photoaging in human skin in vivo and their prevention by all-trans retinoic acid. Photochem. Photobiol. 69, 154–157.

Gilchrest, B.A., 1996. A review of skin aging and its medical therapy. Br. J. Dermatol. 135, 867–875.

Gilchrest, B.A., Yaar, M., 1992. Aging and photoaging of the skin: observations at the cellular and molecular level. Br. J. Dermatol. 127, 25–35.

Hanson, K.M., Clegg, R.M., 2002. Observation and quantification of ultraviolet-induced reactive oxygen species in ex vivo human skin. Photochem. Photobiol. 76, 57–63.

Herron, G.S., Werb, Z., Dwyer, K., Banda, M.J., 1986. Secretion of metalloproteinases by stimulated capillary endothelial cells: II. Expression of collagenase and stromelysin activities is regulated by endogenous inhibitors. J. Biol. Chem. 261, 2814–2819.

Huang, M.T., Ho, C.T., Wang, Z.Y., Ferraro, T., Lou, Y.R., Stauber, K., Ma, W., Georgiadis, C., Laskin, J.D., Conney, A.H., 1994. Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid. Cancer Res. 54, 701–708.

Johnson, L.L., Dyer, R., Hupe, D.J., 1998. Matrix metalloproteinases. Curr. Opin. Chem. Biol. 2, 466–471.

Kobayashi, T., Hattori, S., Nagai, Y., Tajima, S., Nishikawa, T., 1998. Differential regulation of MMP-2 and MMP-9 gelatinases in cultured human keratinocytes. Dermatology 197, 1–5.

Kulms, D., Zeise, E., Poppelmann, B., Schwarz, T., 2002. DNA damage, death receptor activation and reactive oxygen species contribute to ultraviolet radiation-induced apoptosis in an essential and independent way. Oncogene 21, 5844–5851.

Mahato, S.B., Sarkar, S.K., Poddar, G., 1988. Triterpenoid saponins. Phytochemistry 27, 3037–3067.

Maquart, F.X., Bellon, G., Gillery, P., Wegrowski, Y., Borel, J.P., 1990. Stimulation of collagen synthesis in fibroblast cultures by a triterpene extracted from *Centella asiatica*. Connect. Tissue Res. 24, 107–120.

Maquart, F.X., Chastang, F., Simeon, A., Birembaut, P., Gillery, P., Wegrowski, Y., 1999. Triterpenes from *Centella asiatica* stimulate extracellular matrix accumulation in rat experimental wounds. Eur. J. Dermatol. 9, 289–296.

Masaki, H., Atsumi, T., Sakurai, H., 1995. Detection of hydrogen peroxide and hydroxyl radicals in murine skin fibroblasts under UVB irradiation. Biochem. Biophys. Res. Commun. 206, 474–479.

Oikarinen, A., Kylmaniemi, M., Autio-Harmainen, H., Autio, P., Salo, T., 1993. Demonstration of 72-kDa and 92-kDa forms of type IV collagenase in human skin: variable expression in various blistering diseases, induction during re-epithelialization, and decrease by topical glucocorticoids. J. Invest. Dermatol. 101, 205–210.

Qin, J.Z., Chaturvedi, V., Denning, M.F., Bacon, P., Panella, J., Choubey, D., Nickoloff, B.J., 2002. Regulation of apoptosis by p53 in UV-irradiated human epidermis, psoriatic plaques and senescent keratinocytes. Oncogene 21, 2991–3002.

Scharffetter-Kochanek, K., Wlaschek, M., Brenneisen, P., Schauen, M., Bladschun, R., Wenk, J., 1997. UV-induced reactive oxygen species in photocarcinogenesis and photoaging. Biol. Chem. 378, 1247–1257.

Scharffetter-Kochanek, K., Brenneisen, P., Wenk, J., 2000. Photoaging of the skin from phenotype to mechanisms. Exp. Gerontol. 35, 307–316.

Shukla, A., Rasik, A.M., Jain, G.K., Shankar, R., Kulshrestha, D.K., Dhawan, B.N., 1999. In vitro and in vivo wound healing activity of asiaticoside isolated from *Centella asiatica*. J. Ethnopharmacol. 65, 1–11.

Van de Loosdrecht, A.A., Nennie, E., Ossenkoppele, G.J., Beelen, R.H., Langenhuijsen, M.M., 1991. Cell mediated cytotoxicity against U 937 cells by human monocytes and macrophages in a modified colorimetric MTT assay. A methodological study. J. Immunol. Methods 141, 15–22.

Vile, G.F., 1997. Active oxygen species mediate the solar ultraviolet radiation-dependent increase in the tumour suppressor protein p53 in human skin fibroblasts. FEBS Lett. 412, 70–74.

Vile, G.F., Tyrrell, R.M., 1995. UVA radiation-induced oxidative damage to lipids and proteins in vitro and in human skin fibroblasts is dependent on iron and singlet oxygen. Free Radic. Biol. Med. 18, 721–730.

Wenk, J., Brenneisen, P., Meewes, C., 2001. UV-induced oxidative stress and photoaging. Curr. Probl. Dermatol. 29, 74–82.

West, M.D., 1994. The cellular and molecular biology of skin aging. Arch. Dermatol. 130, 87–95.

Yarosh, D.B., Both, D., Brown, D., 2000. Liposomal ursolic acid (merotaine) increases ceramides and collagen in human skin. Horm. Res. 54, 318–321.