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ORIGINAL PAPER

Dawn M. Both · Karina Goodtzova · Daniel B. Yarosh
David A. Brown

Liposome-encapsulated ursolic acid increases ceramides and collagen in human skin cells

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Abstract Skin wrinkling and xerosis associated with aging result from decreases in dermal collagen and stratum corneum ceramide content. This study demonstrated that ursolic acid incorporated into liposomes (URA liposomes) increases both the ceramide content of cultured normal human epidermal keratinocytes (NHEK), and the collagen content of cultured normal human dermal fibroblasts. In addition, URA liposomes increased the ceramide content of the skin of human subjects, with increases in hydroxy ceramides occurring after only 3 days of treatment. Both URA liposomes and retinoic acid decreased markers of keratinocyte differentiation (keratin 1, keratin 10 and involucrin) in cultured NHEK. Thus, URA liposomes have effects on keratinocyte differentiation and dermal fibroblast collagen synthesis similar to those of retinoids. However, this study showed that URA liposomes increase ceramides in NHEK, in contrast to the decreases previously shown to be caused by retinoids. URA liposomes have the potential to be used alone or in combination with other agents to restore or maintain skin ceramide and collagen content.

Keywords Aging · Ursolic acid · Retinoic acid · Ceramide · Collagen

Introduction

During the aging process, skin wrinkling occurs as a result of reductions in collagen in the dermis and epidermal atrophy (Griffiths and Voorhees 1993; Uitto and Bernstein 1998). In addition, reduced barrier function and xerosis occur as a result of reduced stratum corneum thickness and ceramide content (Imokawa et al. 1991; Rogers et al. 1996). Studies of the aging process have shown that ceramides are the lipid class most decreased with age (Rogers et al. 1996). Sun exposure accentuates the skin wrinkling process by suppression of collagen I synthesis and stimulation of collagenase release from inflammatory cells, fibroblasts and keratinocytes (Griffiths and Voorhees 1993; Griffiths et al. 1993).

There are numerous skin-care products addressing the problem of aging skin. The retinoids are among the most well-known of antiaging ingredients. Retinoids exert their effects by engaging nuclear retinoic acid receptors (RAR) thereby altering the expression of genes involved in proliferation and differentiation (Gendimenico and Mezick 1993; Griffiths 1994). Retinoids increase skin thickness and produce an antiwrinkling effect by inducing epidermal keratinocyte hyperplasia (Gendimenico and Mezick 1993). In addition, retinoids produce an antiwrinkling effect by stimulating collagen synthesis in the dermis (Griffiths et al. 1993). However, as well as these beneficial effects, retinoids also cause thinning of the stratum corneum resulting in reduced barrier function and xerosis due to transepidermal water loss (Griffiths 1994; Rawlings et al. 1996). Furthermore, retinoids have been shown to decrease ceramide levels in living skin equivalents (Ponec and Weerheim 1990).

The effects of ursolic acid (URA) on skin physiology were examined in this study, with particular reference to possible antiaging effects. URA (Fig. 1) is a nontoxic naturally occurring triterpenoid that is found in a variety of plants with medicinal properties (Liu 1995). Previous studies have shown that URA has both antiinflammatory and antitumor properties, including the ability to inhibit tumor promotion in mouse skin (Huang et al. 1994; Suh et al. 1998). This study demonstrated that URA also has antiaging properties similar to those of retinoids, including the ability to stimulate collagen production in cultured dermal fibroblasts and to reduce differentiation markers in cultured epidermal keratinocytes. However, in contrast to retinoids, URA stimulates ceramide production in both cultured keratinocytes and human skin. Therefore, unlike retinoids, URA may stimulate antiaging processes without loss of barrier function.

D.M. Both · K. Goodtzova · D.B. Yarosh · D.A. Brown (✉)
AGI Dermatics, 205 Buffalo Avenue, Freeport, NY 11520, USA
e-mail: dbrown@agiderm.com,
Tel.: +1-516-8689026, Fax: +1-516-8689143

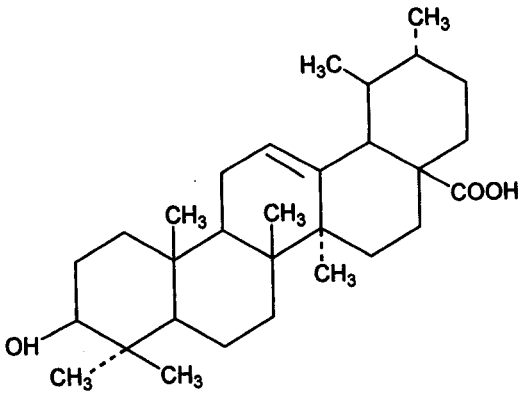


Fig. 1 Structure of URA

Materials and methods

Cell culture

Normal human epidermal keratinocytes (NHEK) were from Clonetics (San Diego, Calif.) and Cascade Biologicals (Portland, Ore.). Normal human dermal fibroblasts (NHDF) were from Clonetics. Both cell types were from multiple neonatal foreskin donors of varied pigmentation. Cells were grown according to the manufacturer's instructions.

Preparation of liposomes and lotions

URA was encapsulated in liposomes using the method of ethanol injection. Briefly, phosphatidylcholine and cholesterol were dissolved in ethanol at a 2.5:1 molar ratio. This solution was then split into aliquots, and a range of concentrations of URA was added so that the loading capacity of liposomes could be determined. One aliquot received no URA to provide an empty liposome control. Each aliquot was injected through a 30G1/2 needle into an equal volume of cold 1× phosphate-buffered saline (PBS). The resulting solutions were subjected to dialysis against 1× PBS to remove ethanol, nonliposomal lipids and unencapsulated URA. The resultant liposomes were analyzed by high-performance thin-layer chromatography (HPTLC) to determine the concentration of URA incorporated into the liposomes. The final concentration of URA in the liposomes used here was 3 m*M*. Lotion was prepared by heating a 1% solution of Carbopol 981 in 1× PBS to 65°C, followed by cooling and addition of triethanolamine to pH 8 to allow gel formation. URA-containing liposomes were incorporated into a lotion at 0.3% and 1% (v/v) with 0.07% Kathon CG added as preservative. All-*trans*-retinoic acid was incorporated into liposomes at 0.83 m*M* using ethanol injection in a procedure identical to that described above for URA.

Treatment of NHEK for examination of ceramide induction

NHEK were cultured until 60–70% confluence and then treated with empty liposomes (vehicle control) or URA liposomes (URA encapsulated in liposomes). For dose response experiments, NHEK were treated with empty liposomes or URA liposomes on days 0, 2 and 4, and harvested on day 6. For the time-course experiment, NHEK were treated with URA liposomes on day 0 and every 3rd day thereafter for the indicated time periods. NHEK harvested after 1, 2 and 3 days were treated only once, those harvested after 5 days were treated twice, and those harvested after 7 and 9 days were treated three times. Treatments were staggered so that cells were harvested on the same day for all treatments.

Lipid extraction from NHEK

Following treatments, cells were harvested using Trypsin/EDTA and Trypsin neutralizing solution (Clonetics or EpiLife) according to the manufacturer's instructions. Cells were counted using a Model ZBI Coulter counter (Hialeah, Fl.), pelleted by centrifugation at 200 *g* for 5 min, washed with 1× PBS, and transferred to a Pyrex tube with a Teflon-lined lid. Lipids were extracted using the procedure developed by Ponec and Weerheim (1990) as follows. The pellet was brought up to 0.375 ml with deionized water (dH_2O), and 2 ml chloroform/methanol (1:2) was added. The suspension was vortexed for 1 min and then mixed end-over-end for 1 h. The pellet was centrifuged at 1000 *g* for 5 min, and the supernatant was saved. The pellet was then extracted with 2 ml chloroform/methanol/dH_2O (1:2:0.5) by vortexing for 1 min, followed by mixing in a shaking water bath for 1 h at 37°C. The pellet was spun down and the resulting supernatant was combined with the first supernatant. The pellet was re-extracted by mixing end-over-end with 2 ml chloroform/methanol (1:2) for 30 min, 2 ml chloroform/methanol (2:1) for 30 min, and then 2 ml chloroform for 15 min. Then, 0.2 ml 2.5% KCl and 2 ml dH_2O were added to the saved supernatants, which were vortexed and mixed end-over-end for 10 min. The tubes were then centrifuged at 1000 *g* for 5 min, and the upper aqueous layer was removed. The aqueous layer was then re-extracted with 4 ml chloroform. The chloroform layers were combined and dried under a stream of nitrogen gas while immersed in a water bath at 50°C. Lipids were dissolved in 200 µl chloroform/methanol (2:1) per 10^6 cells. The lipid solution was then placed into a Pyrex vial with a Teflon-lined lid, and stored under nitrogen gas at –20°C.

Treatment of human subjects

The protocol for this study was approved by the Institutional Review Board at AGI Dermatics. Each subject gave informed consent prior to inclusion in the study. The human subjects were three Caucasian males between the ages of 45 and 55 years. The subjects applied the lotions to the forearms twice per day for 11 days. The subjects were instructed to apply lotion in an amount sufficient to provide full and ample coverage in an area slightly larger than the diameter of a 50-ml polypropylene conical tube (2.5 cm). This was about 20 µl of lotion per treatment.

Lipid extractions from human subjects

Lipids were extracted from the subjects using a modification of the protocol described by Bonté et al. (1997). Following treatment, the area on the forearm to be extracted was first rinsed with tap water, dried thoroughly and then tape-stripped once with Scotch 810 Magic tape. The severed top 1 inch of 50-ml polypropylene conical tubes were used as reservoirs for the solvents during extraction from subjects. The reservoirs were placed and held firmly on the arm and 1 ml cyclohexane/ethanol (4:1) was added and stirred gently for 1 min. Solvent was then removed and placed into a Pyrex tube with a Teflon-lined lid. Then 1 ml cyclohexane/ethanol (1:1) was added to the reservoir, stirred for 1 min, and then removed and added to the tube containing the first extract. The tubes were then dried at 50°C under nitrogen gas as described above. The dried extracts were dissolved in 200 µl chloroform/methanol (2:1). The lipid solution was then placed into a small storage tube with a Teflon-lined lid, purged with nitrogen gas and stored at –20°C.

HPTLC and lipid visualization

Organically bound silica gel HPTLC plates (Techware, catalogue no. Z26529-2) were presoaked for at least 2 h in chloroform/methanol/water (95:20:1) prior to chromatography. Following application of samples and ceramide standards (nonhydroxy ceramides, Sigma, catalogue no. C2137, and hydroxy ceramides, Sigma, catalogue no. C2512) using CAMAG capillary pipettes, the

plate was sequentially placed into four pre-equilibrated HPTLC developing tanks (CAMAG), containing a modified version of the four-solvent development system of Kennedy et al. (1996). The plate was developed to a height of 3 cm from the origin with chloroform/methanol/water (40:10:1), then to a height of 6.5 cm with chloroform/methanol/acetic acid (190:9:1), then to a height of 7.5 cm with hexane/diethyl ether/acetic acid (12:3:2) and finally with petroleum ether to the top of the plate. The plate was allowed to dry thoroughly and was then sprayed with a cupric sulfate/phosphoric acid solution (10% and 8%, respectively). After allowing the plate to air-dry for 5 min, it was placed on a CAMAG plate heater III at 60°C, and the temperature was increased to 160°C over a period of 20 min, or until all lipids were charred and the background began to darken slightly. A photograph was taken of the plates using a Photometrics STAR 1 camera (Bothell, Wash.) and Optimas 5.2 software (Optimas, Tucson, Ariz.). The images were analyzed using BioSoft QuantiScan 2.1 software (Cambridge, UK).

Treatment and procollagen immunohistochemistry in NHDF

NHDF were grown on four-well Lab-Tek chamber slides (Nalgene Nunc International, Naperville, Ill., catalogue no. 177399). Cells were treated with 0.3% and 1% URA liposomes on days 0 and 3, and then fixed for immunohistochemistry in 4% paraformaldehyde in 1× PBS on day 6. Cells were probed with rat anti-human procollagen I, aminoterminal-specific monoclonal antibody IgG_1 (Chemicon International, Temecula, Calif., catalogue no. MAB1912). Labeling was done with anti-rat IgG_1 conjugated with fluorescein isothiocyanate (FITC) (Pharmingen, San Diego, Calif., catalogue no. 10084D). Photographs of the cells were taken and fluorescence intensity was quantified using Northern Eclipse image analysis 2.0 (Empix Imaging).

Treatment and Western blotting of differentiation markers in NHEK

NHEK were grown in 10-cm culture plates (Corning, N.Y.) to 70–80% confluency in low-Ca medium (60 μ*M* Epilife Medium, Cascade Biologicals). Cells were treated daily for 3 days with 0.01% liposomes containing 0.83 m*M* all-*trans*-retinoic acid, 200 μ*M* $CaCl_2$, 1 m*M* dibutyryl cAMP (Calbiochem, catalogue no. 28745), or 1% liposomes containing 3 m*M* URA. The cells were harvested using trypsin/EDTA and trypsin neutralizing solution (EpiLife) according to the manufacturer's instructions. Cells were counted using a Coulter counter, and then pelleted at 200 *g*, and washed with 1× PBS. Pelleted cells were resuspended in 1× sample buffer (Novex, San Diego, Calif.) plus 0.05 *M* DTT (Sigma). The samples were electrophoresed on 10% Tris-glycine SDS-PAGE gels (Novex). Each lane was loaded with 44 μg protein. A second gel was run in an identical fashion and stained with Coomassie so that the Western blotting results could be normalized to protein values. Following electrophoresis, protein was transferred to nitrocellulose (Novex), and the proteins were blotted with antibodies to involucrin (Biomedical Technologies, catalogue no. BT-600), keratin 1 (Enzo Diagnostics, catalogue no. C34904) and keratin 10 (Chemicon International, catalogue no. MAB1605). Involucrin was labeled with an HRP-conjugated anti-rabbit IgG (Biomedical Technologies, catalogue no. BT-604). Keratin 1 and keratin 10 were labeled with anti-mouse IgG_1 supplied with an ECL kit (Amersham, Piscataway, N.J., catalogue no. RPN2108). The blots were photographed using a Photometrics STAR 1 camera and Optimas 5.2 software, and then analyzed using QuantiScan software.

Results

Liposome-encapsulated URA increases ceramides in cultured NHEK

URA was encapsulated into liposomes since it has low solubility in ethanol (<10 m*M*), and URA dissolved in ethanol or other alcohols come out of solution when applied to cultured cells. Moreover, URA solubilized in solvents leaves a powdery residue when applied to human skin, indicative of poor penetration. These difficulties were not encountered with URA liposomes.

Total ceramides were increased approximately threefold by treatment of cultured NHEK for 6 days with URA liposomes at 1% of the cell culture volume (30 μ*M* URA; Fig. 2A). Small increases in ceramides were induced by empty liposomes, but these were not significantly different from the values obtained from control (untreated) NHEK. There were slight variations in basal and induced levels of ceramides between experiments, likely related to passage number (Fig. 2B). Ceramides in untreated or treated NHEK consisted almost entirely of nonhydroxy

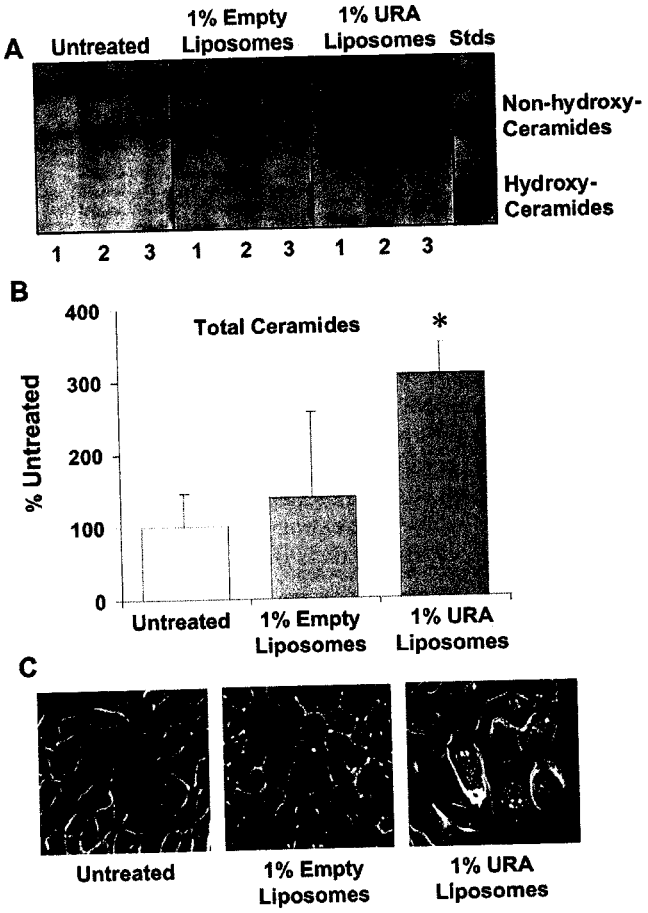


Fig. 2A–C Induction of ceramides in NHEK following treatment with 1% URA liposomes. Cultured NHEK were untreated, or treated with 1% empty liposomes or 1% URA liposomes. Treatments were administered on days 0 and 3, and cells were harvested on day 6. **A** HPTLC results of three different experiments (*lanes 1, 2* and *3*). NHEK in experiments 1 and 3 were at passage 2, and in experiment 2 were at passage 3. **B** Bar graph showing total ceramides as quantified by HPTLC (see **A**) using QuantiScan imaging software (means±SD, n=3; *P<0.05, ANOVA). **C** Photographs of untreated NHEK and NHEK treated with 1% empty liposomes or 1% URA liposomes. Cells treated with 1% URA liposomes were vacuolated (*arrows*) whereas those treated with 1% empty liposomes were not

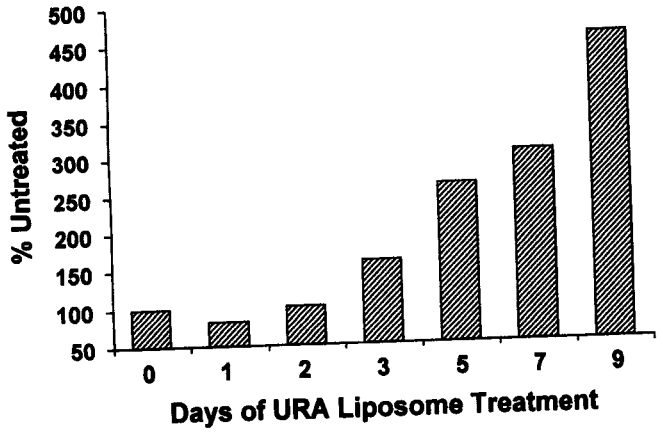


Fig. 3 Time course of ceramide induction by 1% URA liposomes in NHEK indicating that the amounts of ceramides extracted from cultured NHEK treated with 1% URA liposomes increased with the treatment time. Cells were treated on days 0, 3 and 6. All cells were harvested on the same day

ceramides (Fig. 1B). Increases in ceramides coincided with increased vacuolization of URA liposome-treated cells (Fig. 2C). Treatment of NHEK with liposome-encapsulated retinol at 1% of the cell culture volume (25 μM retinol) for 6 days resulted in reductions in ceramides (unpublished data), supporting the findings of Ponec and Weerheim (1990).

A time-course study (Fig. 3) showed that induction of ceramides by URA liposomes was not detectable until 3 days after treatment, at which time increases were approximately 1.6-fold relative to time-zero untreated controls. Thereafter, ceramides were increased 2.5-fold after 5 days of treatment (treated on day 0 and retreated on day 3), 3-fold after 7 days of treatment (treated on days 0, 3 and 6), and 4.5-fold after 9 days of treatment (also treated on days 0, 3 and 6). There was no effect on cell number relative to untreated controls.

Liposome-encapsulated URA incorporated into lotion increases ceramides in skin of human subjects

Treatment of the forearm of human volunteers with lotion containing 0.3% or 1% URA liposomes resulted in induction of ceramides, with increases in hydroxy ceramides generally greater than increases in nonhydroxy ceramides (Fig. 4). In fact, following 3 days of treatment, nonhydroxy ceramides were decreased by approximately 12% following treatment with 0.3% or 1% URA liposomes. In contrast, hydroxy ceramides were increased by approximately 18% following 3 days of treatment with either 0.3% or 1% URA liposomes. Nonhydroxy ceramides correspond to ceramides 1–3 according to the nomenclature of Wertz and Downing (1983), while hydroxy ceramides correspond to ceramides 4–6a, b.

Both nonhydroxy and hydroxy ceramides were increased by approximately 30% following 11 days of treatment with 0.3% URA liposomes. However, at this same time point, nonhydroxy and hydroxy ceramides were increased by only 7% and 18%, respectively, following treatment with 1% URA liposomes. Thus, whereas 1% was the more effective URA liposome concentration for induction of ceramides in cultured NHEK (Fig. 2), 0.3% was the more effective URA liposome concentration for induction of ceramides in human skin. However, it should be noted that whereas cell culture treatments were done only once every 3rd day, treatment of human skin was done twice daily. Thus, the ability of URA liposomes to induce ceramides in human skin may have been saturated by this treatment regimen.

Visual observation of skin during applications indicated no obvious effects on skin morphology, or any irritation of skin.

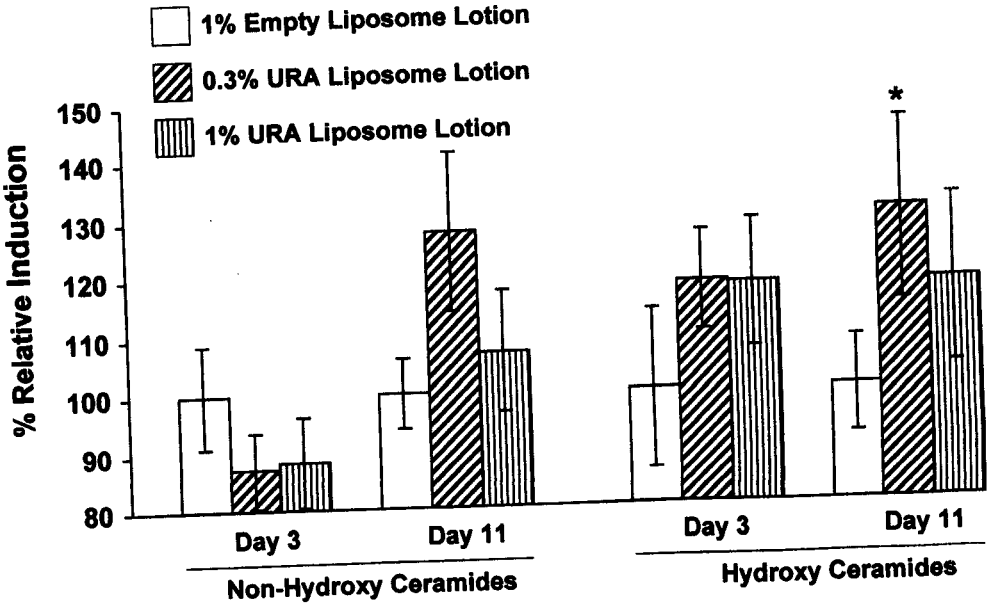


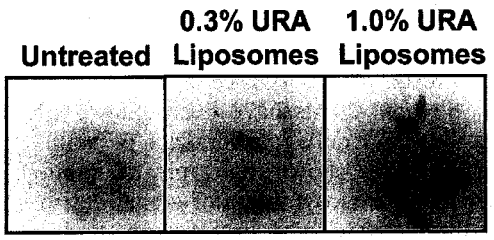
Fig. 4 Induction of ceramides in human skin following treatment with 0.3% or 1% URA liposomes for 3 and 11 days. Subjects applied lotions twice per day for 11 days to designated treatment areas on the forearm with diameters slightly larger than 2.5 cm. Values are relative to those obtained for adjacent areas treated with empty liposome lotion (means±SD, n=3; *P<0.05, ANOVA)

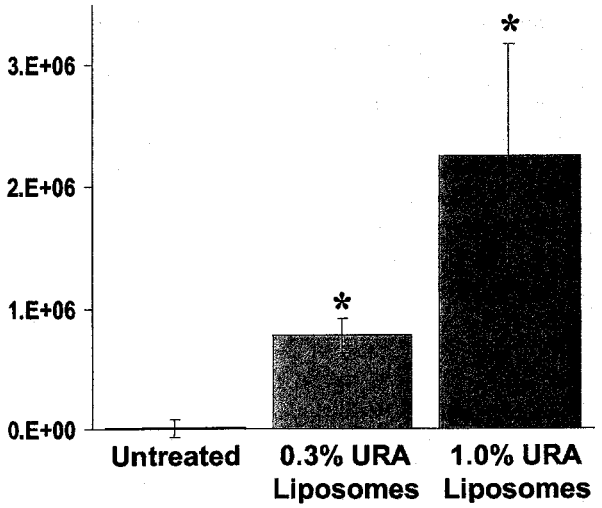
)some-encapsulated URA increases collagen
luction in cultured NHDF

agen production in cultured NHDF was measured as
ollagen, since collagen exists as procollagen until it is
eted into the extracellular matrix (Griffiths et al. 1993).
ollagen was near the limit of detection in untreated
)F, and was increased approximately 70-fold following
ment with 0.3% URA liposomes (Fig. 5). Procollagen
2.9-fold higher in cells treated with 1% URA lipo-
es than in those treated with 0.3% URA liposomes
.5). Smaller increases in procollagen occurred follow-
reatment with empty liposomes (data not shown).

)some-encapsulated URA decreases differentiation
kers in cultured NHEK

ing differentiation of cultured keratinocytes, expres-
of keratin 1, keratin 10, and involucrin increases
idimenico and Mezick 1993). Therefore, markers of
rentiation were examined to determine whether ce-





A, B Induction of procollagen in NHDF following treatment
1% URA liposomes. Cultured NHDF were treated on days 0
, and harvested on day 6. A NHDF fixed and probed with a
llagen antibody followed by a FITC-conjugated secondary
dy. B Bar graph showing cellular procollagen content mea-
as fluorescence intensity for each of the three treatments in
uorescence intensity was quantified using Northern Eclipse
ng software (means±SEM, n=9, 30 and 34; *P<0.05,
VA)

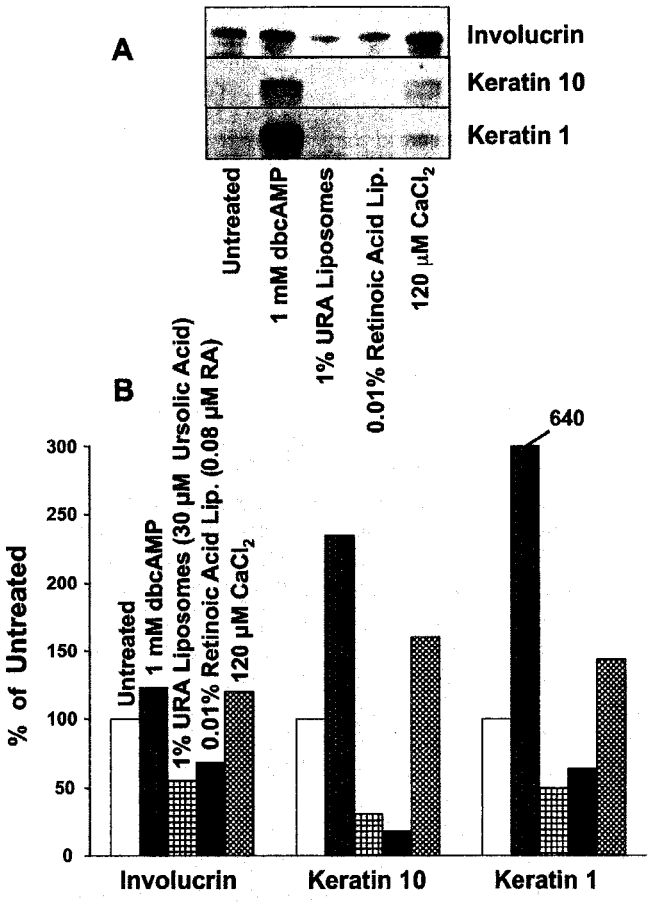


Fig. 6A, B Reduction of differentiation markers in NHEK following treatment with 1% URA liposomes and retinoic acid liposomes. Cultured NHEK were treated with the indicated treatments for 3 days, with daily replenishment of treatment. Following treatment, cell extracts were subjected to Western blotting. Dibutyryl cAMP and $CaCl_2$ were used as positive controls for induction of involucrin, keratin 1 and keratin 10. **A** Western blots showing levels of these proteins in cell extracts. **B** Bar graph showing levels of each protein for each treatment. The protein in each lane was quantified by normalization to the protein in similarly run SDS-PAGE Coomassie-stained gels, using QuantiScan imaging software

ramides induced by URA liposomes were a simple by-product of keratinocyte differentiation, or were independent of keratinocyte differentiation. Rhodamine B staining (Delescluse et al. 1976) in preliminary experiments showed that treatment of NHEK with 1% URA liposomes for 4 days resulted in a 46% decrease in keratinization (unpublished data). Subsequent experiments were done using Western blotting. As positive controls for differentiation, NHEK were treated with 1 m*M* dibutyryl cAMP or 120 μ*M* $CaCl_2$ (Fig. 6). These agents are known to induce keratinocyte differentiation (Delescluse et al. 1976), with concomitant induction of keratin 1, keratin 10, and involucrin expression (Fuchs 1993). Whereas both dibutyryl cAMP and $CaCl_2$ induced these markers of keratinocyte differentiation, both 0.8 μ*M* retinoic acid administered as 0.01% retinoic acid liposomes, and 30 μ*M* URA administered as 1% URA liposomes, resulted in decreases. Thus, although reductions in ceramides are associated with

NHEK dedifferentiation induced by retinoids, increases in ceramides are associated with dedifferentiation induced by URA liposomes.

Discussion

This study demonstrated that URA liposomes produce changes in human skin cells that are indicative of antiaging effects. Two primary responses were noted. First, URA liposomes increased the ceramide content of both cultured NHEK (Figs. 1 and 2), and skin of human subjects (Fig. 4). Second, URA liposomes increased the collagen content of cultured NHDF (Fig. 5). In a recent study using human skin, URA has also been found to stimulate collagen synthesis (Nishimori et al. 1999). Reductions in both ceramides and collagen are known to occur as a result of the aging process (Griffiths and Voorhees 1993; Rogers et al. 1996). Reductions in ceramides are associated with reduced barrier function, and dry flaky skin (Imokawa et al. 1991; Rogers et al. 1996). Reductions in collagen content are associated with loss of tensile strength and development of skin wrinkling (Griffiths and Voorhees 1993). These changes occur not only as a result of aging, but also as a result of sun exposure. Thus, application of URA liposomes to human skin would be expected to counteract the effects of both aging and photodamage.

Whereas induction of ceramide synthesis is usually associated with keratinocyte differentiation, this study showed that induction of ceramides by URA liposomes occurs in the absence of keratinocyte differentiation. In fact, treatment of NHEK with URA liposomes resulted in a clear reduction of markers of keratinocyte differentiation, including keratin 1, keratin 10, and involucrin (Fig. 6). In this respect, URA liposomes exert effects on NHEK similar to those of retinoids. In the case of retinoids, reductions in these markers of differentiation in cell culture studies have been linked to epidermal thickening in skin with resultant reduction of wrinkling (Gendimenico and Mezick 1993). However, unlike retinoids, which decrease ceramide synthesis (Ponec and Weerheim 1990), URA liposomes stimulate ceramide synthesis. Thus, URA liposomes may have the potential to stimulate both epidermal thickening and barrier function.

Previously, it has been shown that NHEK grown under submerged conditions produce virtually no ceramides (Boelsma et al. 1999; Ponec et al. 1988). Indeed, in this study, only one ceramide species was prominent in untreated NHEK (Fig. 2B). However, following treatment with URA liposomes, ceramide profiles were similar to those found in previous studies in which a stratified epidermis formed due to growth on a de-epidermized dermis at the air-liquid interface for 1 to 2 weeks (Ponec and Weerheim 1990; Ponec et al. 1988; Ponec et al. 1997). This comparison further reinforces the finding that NHEK treated with URA liposomes produce ceramides indicative of differentiated cells, even though URA liposomes induce protein markers of keratinocyte dedifferentiation.

Although URA liposome treatment for both 3 and 11 days resulted in increases in hydroxy ceramides in human skin, nonhydroxy ceramides showed a clear trend towards decreases following 3 days of treatment (Fig. 4). This result may be commensurate with the known metabolism and function of ceramides. A previous study has shown that the flow of carbon during ceramide synthesis is from glucosylceramides to nonhydroxy ceramides, and then to hydroxy ceramides (Hedberg et al. 1988). Hydroxy ceramides are then linked to involucrin via its numerous glutamate residues during the process of cornification (Swartzendruber et al. 1987). Our results lead us to suggest that following 3 days of treatment, nonhydroxy ceramides may have been depleted to provide for the synthesis of hydroxy ceramides. Following 11 days of treatment, nonhydroxy ceramides may have been replenished and further expanded so that both nonhydroxy and hydroxy ceramides were elevated.

Stratum corneum ceramides decrease by approximately 10–15% per decade after the age of 20 years (Imokawa et al. 1991; Rogers et al. 1996). This study showed that treatment with lotion containing 0.3% URA liposomes for 11 days increased both nonhydroxy and hydroxy ceramides by approximately 30% (Fig. 4). Thus, the increases in ceramides induced by URA liposomes in this study are equivalent to the losses that would be incurred as a result of two to three decades of aging.

Several agents have been shown to increase the ceramide and collagen contents of skin. Rawlings et al. (1996) have shown that topically applied lactic acid increases the biosynthesis of ceramides in human skin, resulting in reductions in transepidermal water loss and xerosis. Vitamin C treatment results in increases in glucosylceramides and ceramides in NHEK grown at the air-liquid interface (Ponec et al. 1997). Vitamin C also stimulates dermal collagen synthesis and is a cofactor for collagen synthesis (Pinnell 1985). Estrogen replacement therapy has long been known to increase collagen deposition in skin (Brincat et al. 1987; Varila et al. 1995), although there is at least one dissenting study (Haapasaari et al. 1997). While retinoids may negatively affect skin barrier function, they dramatically improve the appearance of photoaged skin by promoting collagen synthesis in the dermis (Griffiths et al. 1993). As noted in this study, URA liposomes increase both the collagen and ceramide content of skin cells. Each of these agents has its own advantages, and in some cases, combinations of agents may prove to be most beneficial.

The overlapping but divergent modes of action of retinoids and URA observed in this study may reflect interactions between their respective receptor systems. Evidence to date indicates that these molecules exert effects by binding to the steroid/thyroid/retinoid/PPAR (peroxisome proliferator-activated receptor) nuclear receptor superfamily. There is evidence suggesting that URA downregulates metalloproteinase-9 via binding to the glucocorticoid receptor (Cha et al. 1998). In addition, a synthetic analog of oleanolic acid (closely related to URA) has been shown to stimulate adipocyte differentiation via binding

to PPAR-γ (Wang et al. 2000). Induction of both PPAR-α and PPAR-γ has been linked to differentiation of human keratinocytes (Rivier et al. 1998), and induction of PPAR-α has been linked to increased synthesis of ceramides (Rivier et al. 2000). Retinoids stimulate differentiation via RARs and retinoid X receptors (RXRs). Both RARs and PPARs can heterodimerize with RXRs, but they have differing effects on patterns of gene expression and cellular differentiation (DiRenzo et al. 1997). We are pursuing further studies to determine how URA affects these interlinked signaling pathways.

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