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ORIGINAL ARTICLE

Retinyl retinoate induces hyaluronan production and less irritation than other retinoids

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ABSTRACT

Hyaluronan, a non-sulfated glycosaminoglycan, retains water, maintains the extracellular spaces and facilitates the transport of ion solutes and nutrients. Hyaluronan is closely involved in keratinocyte proliferation, migration and differentiation. The synthesis of hyaluronan *in vitro* can be stimulated by several growth factors, including retinoids, dibutyryl cyclic adenosine monophosphate and peroxisome proliferator-activated receptor-α agonist. In this study, we examined retinyl retinoate (a novel retinol derivative) on hyaluronan expression in primary human keratinocytes and in hairless mouse epidermal skin. Histochemistry using hyaluronan-binding protein revealed that topical retinyl retinoate increased the intensity of hyaluronan staining in murine skin. Moreover, topical retinyl retinoate increased CD44 (hyaluronan receptor) expression. Using reverse transcription polymerase chain reaction, we assessed the expression level of the hyaluronan synthase 2 (*HAS2*) gene in primary human keratinocytes and in hairless mouse epidermal skin. We found that retinyl retinoate upregulated mouse *HAS2* and human *HAS2* mRNA. Application of retinyl retinoate induced increasing transepidermal water loss less than retinol, retinoic acid and retinaldehyde. Taken together, we suggest that retinyl retinoate is more effective on hyaluronan production and less of an irritant than other retinoids.

Key words: hyaluronan, hyaluronan synthase 2, retinyl retinoate, transepidermal water loss.

INTRODUCTION

Hyaluronan is a high molecular mass glycosaminoglycan composed of alternating D-glucoronic acid and N-acetyl-D-glucosamine residues and is a major component of the extracellular matrix (ECM).[1] It is well known that hyaluronan retains water, maintains the extracellular space, and facilitates the transport of ion solutes and nutrients.[2,3] Hyaluronan is involved in a wide range of cellular functions including cell proliferation and migration, wound repair, cell locomotion and tumor invasion.[4,5] Keratinocytes and fibroblasts provide the principal source of hyaluronan in the skin. The binding of hyaluronan to CD44 stimulates keratinocyte differentiation, cholesterol synthesis, lamellar body formation/secretion and permeability homeostasis.[6]

Unlike other glycosaminoglycans, hyaluronan is synthesized in the inner space of the plasma membrane by hyaluronan synthases (HAS1, HAS2, HAS3).[7] HAS2 is a major producer of hyaluronan in the epidermis.[8] The synthesis of hyaluronan *in vitro* has been shown to be stimulated by several growth factors, retinoids and dibutyryl cyclic adenosine monophosphate (AMP).[9]

Retinol and its derivative have been widely used for dermatological applications and cutaneous malignancies. In particular, retinoids have been used for

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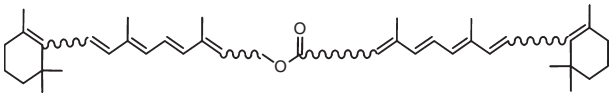

Figure 1. Structure of retinyl retinoate.

cosmetic formulations to reduce wrinkles and improve cellulite.[10–12] However, despite many beneficial effects, the topical application of retinoids often causes severe local irritation manifested as mild erythema and stratum corneum peeling of the skin. The erythematous reaction is clinically similar to a mild irritant dermatitis.[13,14] For this reason, it is important to develop new retinol derivatives that have not only the same activity for deterring skin aging as vitamin A, but also reduced skin-irritant properties.

A retinol derivate, retinyl retinoate (Fig. 1), was synthesized with a condensing reaction between retinol and retinoic acid to improve its photo-stability, and an analysis of its *in vitro* biological activity revealed that it had enhanced thermal stability and decreased photosensitivity, and it exhibited decreased cell viability when compared to retinol.[15]

The aims of this study were to determine effects of retinyl retinoate (a novel retinol derivative) on hyaluronan expression and to compare irritation of retinyl retinoate with other retinoids.

METHODS

Experimental protocols and functional studies

Female hairless mice (Skh1/Hr), approximately 6–8 weeks old, were fed a standard mouse diet and water ad libitum. Groups of animals were topically treated with one of the following agents once daily for 3–5 days: 0.05% retinaldehyde (Sigma Chemical, St Louis, MO, USA), 0.05% retinyl retinoate (Enprani, Incheon, Korea), 0.05% all-trans retinoic acid (Sigma Chemical), 0.05% retinol (Sigma Chemical) or vehicle only (propylene glycol : ethanol = 7:3 v/v) for the control. Transepidermal water loss (TEWL) was measured before and daily for 3 days with an evaporation meter (Tewameter TM 210; Courage and Khazaka, Cologne, Germany).

Immunostaining of hyaluronan and CD44

Tissue was fixed in 10% formaldehyde, embedded in paraffin and processed for histological analysis. Sections were cut to 4 μm, mounted onto slides, deparaffinized and rehydrated in a graded ethanol series. Endogenous peroxidase was inactivated with 3% hydrogen peroxide in Tris-buffered saline (TBS). Antigen de-masking was performed by subjecting the sections to microwave treatment in Target Retrieval Solution (DakoCytomation, Carpentaria, CA, USA). The sections were incubated in Protein Block (DakoCytomation) for 20 min and incubated with 5 μg/mL of biotinylated hyaluronic acid binding protein (HABP) (Seikagaku Kogyo, Tokyo, Japan) or anti-CD44 (BD Biosciences Pharmingen, San Diego, CA, USA) in Dako diluent at 4°C overnight. The slides were incubated with streptavidin peroxidase (DakoCytomation) or horseradish peroxidase-conjugated goat anti-rat immunoglobulin G (Santa Cruz Biothechnology, Santa Cruz, CA, USA) at room temperature for 30 min, washed again with the buffer and incubated for 5 min in 3,3′-diaminobenzidine (DAB) solution (DakoCytomation). Finally, the sections were washed in distilled water and subjected to Mayer's hematoxylin staining.

RNA isolation from epidermis

To separate the epidermis from the underlying dermis, skins were floated on 10 mmol/L ethylene diamine tetra acetate at 37°C for 30 min after which the epidermis could be pulled away from the underlying tissue with forceps. RNA was isolated from single nuclei using RNAzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Cell culture

Normal human epidermal keratinocyte (NHEK) (Cascade Biologics, Portland, OR, USA) was routinely grown in Epilife medium (Cascade Biologics) with human keratinocyte growth supplement (HKGS) (Cascade Biologics). The culture medium was changed every 2–3 days.

Assay for hyaluronan

Once the cells reached confluency, they were seeded in six-well plates (1.0×10^5 cells/well). After 2 days, the cells were incubated for 24 h with one of the following: retinaldehyde (1 μmol/L) and retinyl retinoate (1 μmol/L) in HKGS-free Epilife medium. At the end of incubation, the medium was collected and centrifuged at 15 000 *g* for 5 min; and supernatants were analyzed for hyaluronan using an enzyme-linked

immunosorbent assay (ELISA) kit (Echelon Bioscience, Salt Lake, UT, USA).

RNA isolation form primary keratinocyte cells

Once the cells had reached confluency, they were seeded in a 100 π dish (1.0×10^6 cells/well). After 2 days, the cells were incubated for 6 h with solvent retinaldehyde (1 μmol/L) and retinyl retinoate (1 μmol/L). RNA was isolated from single nuclei using RNAzol (Invitrogen) according to the manufacturer's instructions.

Reverse transcription polymerase chain reaction (RT–PCR)

The mRNA levels were analyzed by RT and subsequent PCR. RT–PCR was performed using an RNA PCR Kit (AMV) ver. 2.1 (Takara, Kyoto, Japan) according to the manufacturer's instructions with the following modifications: the mixture was incubated for 30 min at 10°C to extend the primer for effective annealing followed by RT for 1 h at 42°C. The reaction was stopped by heating for 5 min at 95°C and subsequent cooling on ice. The following primers were designed for RT–PCR: sense 5′-ACA GGC ACC TTA CCA ACA GGG TGT-3′ and antisense 5′-GCA TGC ATA GAT CAA AGT TCC CAC G-3′ for mHas2; sense 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′ and antisense 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′ for mβ-actin; sense 5′- ATG TAC ACA GCC TTC AGA GC -3′ and antisense 5′- TCG CTT CGT AGG TCA TCC AC -3′ for hHas2; and sense 5′-GTG GGG CGC CCC AGG CAC CA-3′ and antisense primer 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′ for hβ-actin. PCR was performed with 1.25 U of Ex Taq (Takara, Shiga, Japan) in Ex Taq buffer (Takara) containing 0.2 mmol/L deoxyribonucleotide triphosphates. Amplification was carried out in a DNA Engine DYAD PCR machine (MJ Research, Waltham, MA, USA).

RESULTS

Effect of TEWL in hairless mouse skin by topical application of retinoids

Transepidermal water loss (a marker of cutaneous barrier function) was used in monitoring the degree of skin irritation. TEWL value of hairless mouse skin was normally 5–10 mg/cm^2 per h. Topical application of retinoids increased TEWL values. After 3–5 days of retinyl retinoate application, TEWL value was increased. Other retinoids showed strong signs of irritation after 2 days. So, TEWL values of retinol, retinoic acid and retinaldehyde were higher than that of retinyl retinoate (Fig. 2). The ranking order of retinoid-like irritancy is as follows: retinyl retinoate < retinol < retinoic acid = retinaldehyde. In previous

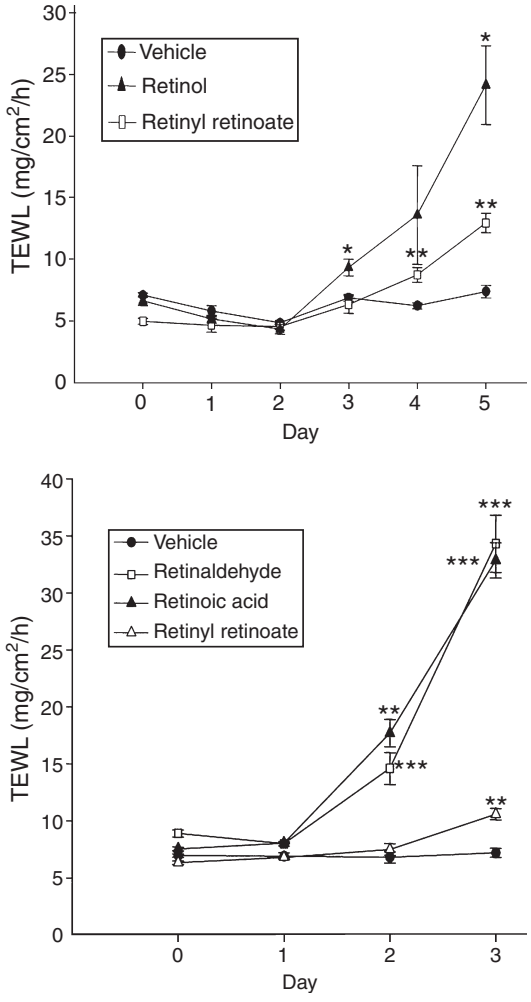

Figure 2. Effect on transepidermal water loss (TEWL) by topical application of retinoids in hairless mouse. Groups of mice (n = 4) were topically treated with retinoids once daily for 3–5 days. Retinol, retinaldehyde, retinoic acid and retinyl retinoate were administrated at the same dose 0.05%, and propylene glycol : ethanol (7:3) was used as vehicle-treated control. Then, the TEWL value was measured in hairless mouse dorsal skin. Bars represent the mean ± standard error of the mean. Asterisks indicate a significant difference compared with the baseline, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

data, cell toxicity[15] and human primary patch test results (data not shown) showed in accordance with TEWL of hairless mouse dorsal skin. Therefore, retinyl retinoate induced less skin barrier disruption than retinol, retinoic acid and retinaldehyde, but had retinoid-like activity.

Effect of retinyl retinoate on hyaluronan expression in hairless mouse skin

The effect of retinyl retinoate on hyaluronan expression was compared to that of retinol, all-trans retinoic acid and retinaldehyde for 3 days. Histochemical analysis showed marked binding of HABP to the epidermis and dermis in retinoid-treated mice when compared with vehicle-treated skin used as a control. Similar to other retinoid, retinyl retinoate induced hyaluronan expression. Retinaldehyde treatment resulted in a slightly greater increase in hyaluronan expression than treatment with retinol or all-trans retinoic acid (Fig. 3), so we used retinaldehyde as a positive control in the following experiments.

Effect of retinyl retinoate on CD44 expression in hairless mouse skin

To determine whether the retinyl retinoate affects CD44 expression in the epidermis of mouse skin, we performed CD44 staining. CD44 is a predominant receptor for hyaluronan on the cell surface of keratinocytes. Topical application of 0.05% retinyl retinoate for 3 days resulted in increased expression of CD44 protein (Fig. 4) in the follicular and interfollicular epidermis.

Effect of retinyl retinoate on mRNA expression of *HAS2* in hairless mouse epidermis

We investigated the possible involvement of the *HAS2* gene in the stimulation of hyaluronan production. After topical applications of retinyl retinoate, total

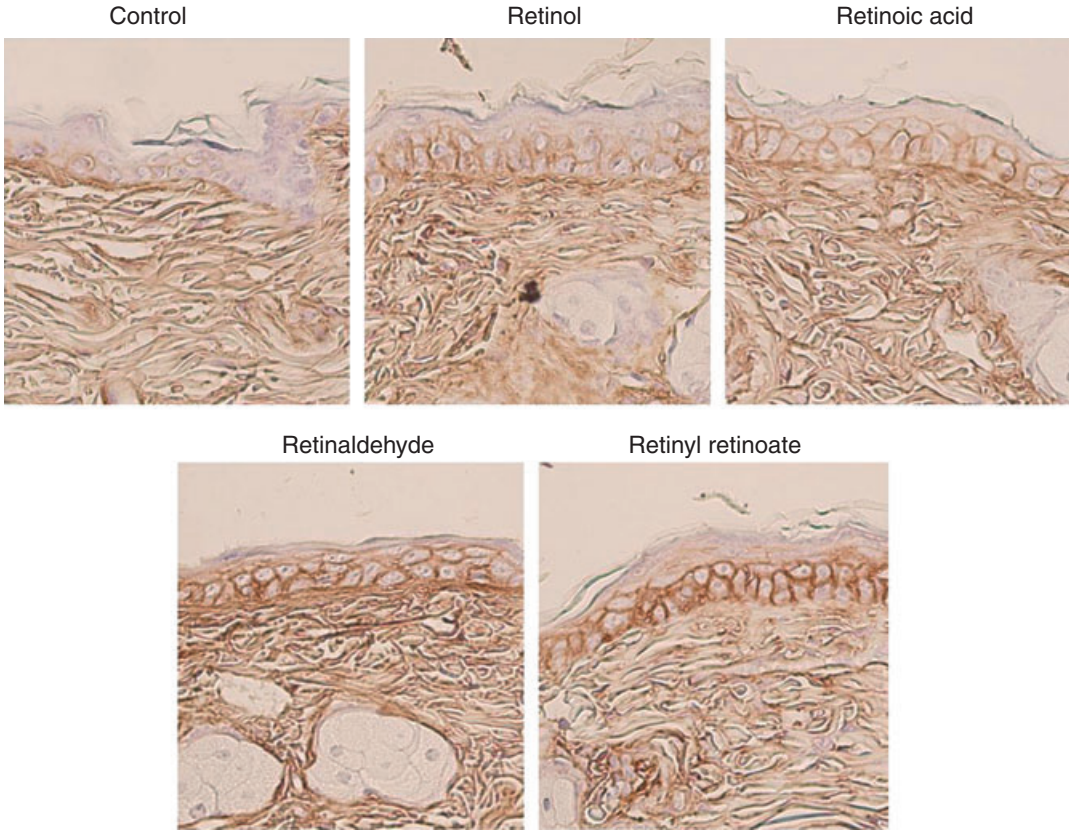

Figure 3. Expression of hyaluronan is enhanced after treatment with topical retinyl retinoate. Retinoids and vehicle were applied once daily at a dose of 0.05% on the hairless mice skin for 3 days. Histological sections of mice skins were stained with hyaluronic acid binding protein (HABP) after ×400 magnification.

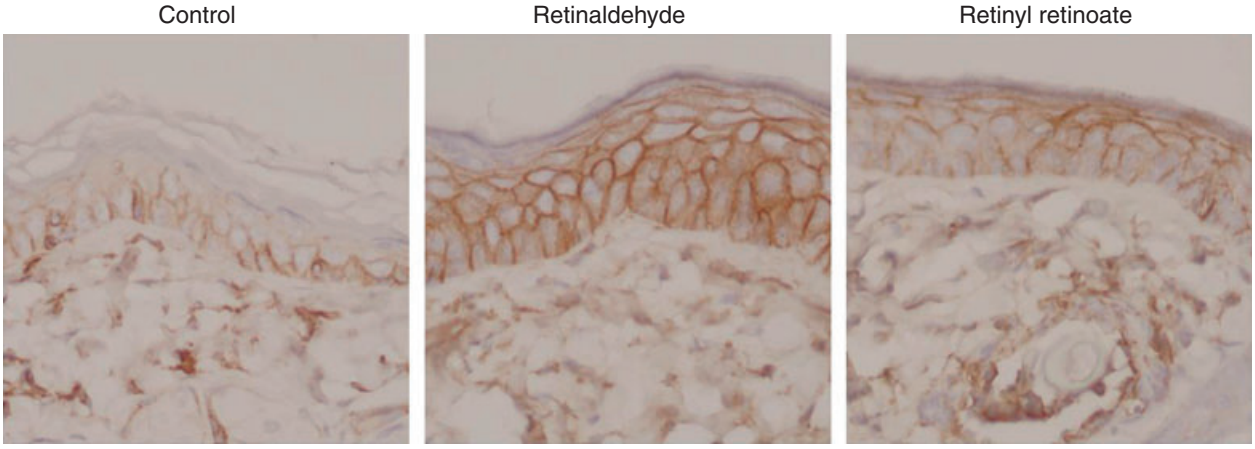

Figure 4. Expression of CD44 is enhanced after topical retinyl retinoate treatment. Retinaldehyde, retinyl retinoate and vehicle were applied once daily at a dose of 0.05% on the hairless mice skin for 3 days. Histological sections of mice skins were stained with anti-CD44 antibody (CD44) after ×400 magnification.

RNA was extracted from the epidermis for RT–PCR analysis. Retinaldehyde and retinyl retinoate increased the expression of *HAS2*. The data were normalized with β-actin (Fig. 5).

Induction of hyaluronan in human primary keratinocyte

Figure 6 shows the effect of retinyl retinoate treatment on the expression of hyaluronan in human primary keratinocytes. The hyaluronan content in the culture was stimulated by retinaldehyde and retinyl retinoate. Treatment of retinaldehyde and retinyl retinoate resulted in a 7.6- and 7.8-fold increase in hyaluronan amounts, respectively (Fig. 6a). We next used RT–PCR to assess the amount of *HAS2* mRNA produced. HAS2 is a major producer of hyaluronan in the epidermis. Retinyl retinoate stimulated human *HAS2* mRNA (Fig. 6b).

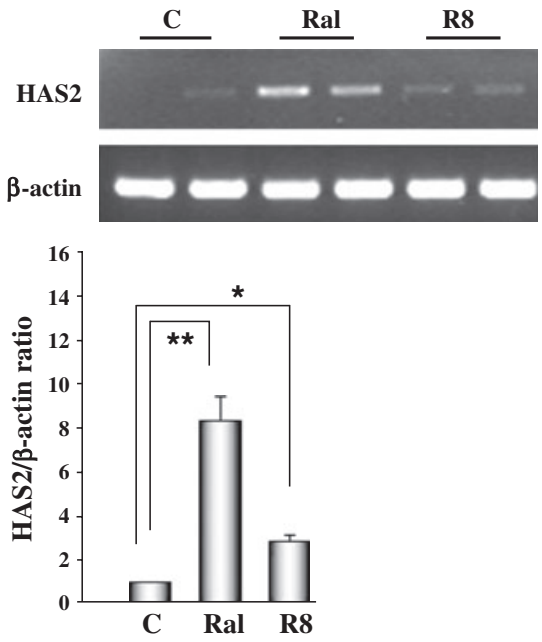

Figure 5. *HAS2* expression is induced in hairless mouse skin after retinyl retinoate. Retinaldehyde and retinyl retinoate were applied once daily for 3 days. The mRNA levels of *HAS2* were determined by reverse transcription polymerase chain reaction. The data were normalized with β-actin. Asterisks indicate a significant difference compared with the control or other group, $^{*}P < 0.05$, $^{**}P < 0.01$. C, control; Ral, retinaldehyde; R8, retinyl retinoate.

DISCUSSION

In previous histochemical investigations, the presence of abundant hyaluronan has been demonstrated in dermal tissue as well as in the intercellular space of granular, spinous and basal cells in the epidermis of human skin.[16,17] The hyaluronan content of human skin is known to decrease with age. In particular, epidermal hyaluronan diminishes almost completely with time, resulting in the dryness associated with aged skin.[18] This creates problems in wound healing and other inflammatory diseases involving the skin.[19] Moreover, hyaluronan, an effective scavenger of free radicals, may also serve a protective role in the epidermis by scavenging reactive oxygen species generated by ultraviolet

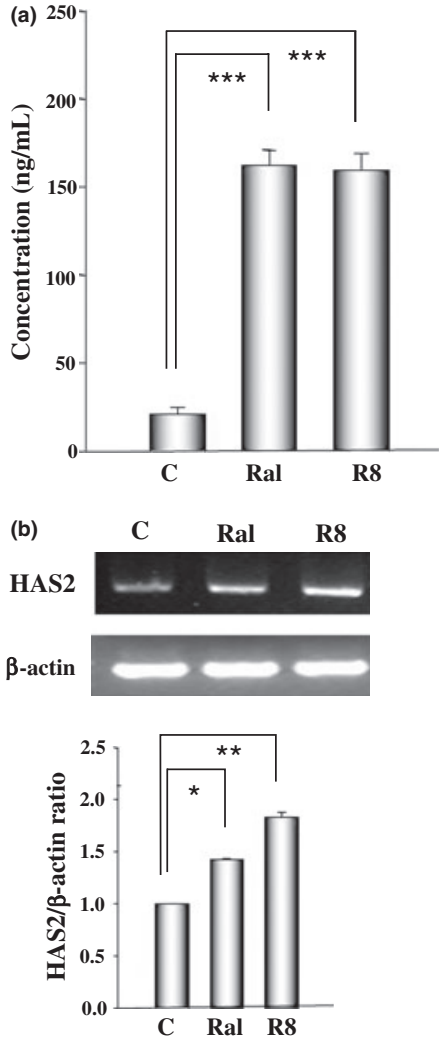

Figure 6. Retinyl retinoate induced higher levels of hyaluronan in cultured human primary keratinocyte. (a) Primary human keratinocytes were cultured for 2 days and incubated for 24 h in the presence of retinaldehyde (1 μmol/L) and retinyl retinoate (1 μmol/L). Supernatants were analyzed for the presence of hyaluronan using an enzyme-linked immunosorbent assay kit. Asterisks indicate a significant difference compared with the control or other group, ***$P < 0.001$. (b) Primary human keratinocytes were cultured for 2 days and incubated for 6 h in the presence of retinaldehyde (1 μmol/L) and retinyl retinoate (1 μmol/L). The mRNA expression of *HAS2* was determined by reverse transcription polymerase chain reaction. Asterisks indicate a significant difference compared with the control or other group, *$P < 0.05$, **$P < 0.01$. C, control; Ral, retinaldehyde; R8, retinyl retinoate.

radiation.[20] The rapid turnover of hyaluronan may help to remove and clear noxious compounds from the epidermis.[4]

In this study, we found that retinyl retinoate, a novel retinol derivative, stimulates hyaluronan production in hairless mouse epidermal skin and human primary keratinocytes (Figs 3,6a, respectively). The medium from retinoate-treated cells showed more hyaluronan production when compared with controls in human primary keratinocyte.

The predominant receptor for hyaluronan on the cell surface of keratinocyte is CD44.[21,22] Hyaluronan is often anchored to CD44, an ubiquitous, abundant and functionally important surface receptor that displays hyaluronan-binding site. The binding of hyaluronan to CD44 causes cells to adhere to ECM components and has also been implicated in the induction of multiple cellular functions.[23]

Retinyl retinoate stimulates CD44 expression in hairless mouse epidermal skin like retinaldehyde (Fig. 4). Enhanced hyaluronan production after topical treatment topical retinyl retinoate is due to upregulation of *HAS2* in hairless mouse skin (Fig. 5) and in human primary keratinocytes (Fig. 6b).

These findings suggest that *HAS2* gene expression plays an important role in regulating hyaluronan synthesis in response to retinyl retinoate in the epidermis.

In a prior study, we reported that retinyl retinoate is suitable for use as an anti-wrinkle agent in cosmetics based on *in vitro* biological assays because it demonstrates enhanced thermal stability and decreased photosensitivity, and exhibits decreased cell toxicity compared to retinol.[15] Retinyl retinoate induced less skin barrier disruption than retinol, retinoic acid and retinaldehyde, but had retinoid-like activity (Fig. 2). The ranking order of retinoid-like irritancy is as follows: retinyl retinoate < retinol < retinoic acid = retinaldehyde.

In conclusion, the findings presented here demonstrate that retinyl retinoate is comparable to well-known retinoids in its ability to induce hyaluronan production through induction of *HAS2* expression, and does not induce skin irritation, compared to other retinoids. Therefore, retinyl retinoate has potential for future use as a cosmetic ingredient.

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