Safety Evaluation and Anti-wrinkle Effects of Retinoids on Skin

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Retinoids have many beneficial effects on dermatological applications. But, retinoids cause skin irritation. In this study, the safety of retinoids was clarified via both primary skin irritation test in rabbits and sensitization study using an integrated model for the differentiation of chemical-induced allergic and irritant skin reaction (IMDS), an alternative method to sensitization test. The effects of retinoids on the change of ultraviolet A (UVA)-induced matrix metalloproteinase-1 (MMP-1) in human skin fibroblasts and the modulation of type-1 pN collagen synthesis in hairless mice were examined to clarify the anti-wrinkle effects. All-*trans* retinol (t-ROL) and its derivative, all-*trans* retinoic acid (t-RA), showed mild skin irritation but did not induce the sensitization. t-ROL and t-RA exerted anti-wrinkle effects by inhibiting the UVA-induced MMP-1 in human skin fibroblasts and increasing the type-1 pN collagen synthesis in hairless mice. These findings suggest that retinoids do not induce the allergy, and show anti-wrinkle effects by decreasing MMP-1 activation and increasing collagen synthesis.

Key words: Retinoids, Primary skin irritation, Sensitization, IMDS, MMP-1, Type-1 pN collagen

INTRODUCTION

Retinoids, such as retinol and retinoic acid, have been increasingly used for topical and systemic application. However, they induce mild erythema on the skin. It is not clear whether the redness induced by retinoid is caused by the sensitization or irritation. Generally, retinoids are considered to have no allergic potentials. However, since clinical and histological features of allergic and irritant contact dermatitis are similar, it is difficult to estimate the allergic potential of retinoid using guinea pig maximization test (GPMT) which is usually used. Recently, the alternative methods to the sensitization such as local lymph node assay (LLNA) and an integrated model for the differentiation of chemical-induced allergic and irritant skin reaction (IMDS) have been studied and developed by many researchers. And validation studies on the alternative methods to the sensitization are in progress (Blotz *et al.*, 2000; Loveless *et al.*, 1996; Ulrich *et al.*, 1998; Vohr *et al.*, 1994, 2000). The IMDS (Homey *et al.*, 1998) is a new method amending the defects of LLNA that uses the isotopes. Allergy is based on the skin immunological responses that result in the induction of antigen-specific memory T cells, while irritation is

defined to be non-immunological skin reactions that show predominantly skin inflammation (Homey *et al.*, 1998). The allergic potential of retinoids is considered to be easily estimated by IMDS that is established by the mechanisms underlying both irritant skin reactions and the induction of contact allergy.

Matrix metalloproteinase-1 (MMP-1) is one of three collagenases that can degrade the interstitial collagen types I, II, and III at neutral pH. As these collagens are the most abundant proteins in the body, collagenases play a critical role in modeling and remodeling of the extracellular matrix (Kim *et al.*, 2006). It has been known that retinoids affected the expression of many genes involved in embryonic development, cell differentiation, and homeostasis. One important target gene for retinoids is MMP-1 (collagenase), the only enzyme active at neutral pH that can degrade interstitial collagen, a major component of extracellular matrix (Pan *et al.*, 1995). These results suggested that retinoids could play a role in anti-wrinkle effects. Retinoids also may be effective in cancer therapy by blocking MMP synthesis which affects decrease of tumor invasiveness (Sakabe *et al.*, 2007). Retinoids have been shown to stimulate the deposition and synthesis of collagen in the subepidermal dermis of photoaged hairless mice (Chen *et al.*, 1992; Schwartz *et al.*, 1991) and human (Griffiths *et al.*, 1993; Wang *et al.*, 2002).

In this study, we clarified the safety of all-*trans* retinol (t-ROL) and all-*trans* retinoic acid (t-RA) based on primary skin irritation in rabbits and sensitization using IMDS. We

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examined the effects of retinoids on the change in UVA-induced matrix metalloproteinase 1 (MMP-1) in human skin fibroblasts and the modulation of type-1 pN collagen synthesis in hairless mice to clarify the anti-wrinkle effects.

MATERIALS AND METHODS

Primary skin irritation test. Skin irritation potencies of t-ROL and t-RA were assessed according to Draize test (1959) with a slight modification. Approximately 24 hours prior to application of the test substance, the hair of New Zealand white rabbits (6 animals per group, Samtako Bio Korea) was removed from the dorso-lumbar region taking care not to damage the skin. t-ROL (0.1%) or t-RA (0.1%) were applied to intact or scratched skin for longer than 4 hours. The grades of treated sites were made at least 24, 48 and 72 hr after treatment of test substance using the Draize scores.

The estimation of sensitization potential using IMDS method. t-ROL was obtained from Sigma-Aldrich (St Louis, MO, USA), and dissolved in "DAE 433", a mixture of 40% dimethylacetamide, 30% acetone and 30% ethanol as described by Maurer *et al.* (1980). Dinitrochlorobenzene (DNCB, 1%) was used as positive control.

Previous studies showed that optimal sensitization and hapten-induced activation of skin-draining lymph node cells were obtained after sensitizer treatment for 3 consecutive days (Gerberick *et al.*, 2007; Ulrich *et al.*, 1998; Vohr *et al.*, 1994). Therefore, five female BALB/c mice per group were topically treated on the dorsal surfaces of both ears with 50 µ*l* of retinol (2%, 1% or 0.1%), or vehicle (DAE 433) alone for 3 consecutive days. On day 0 and 3, ear thickness was measured using Digimatic Indicator (Mitutoyo, Japan) and mean ear swelling was calculated. Afterward, mice were sacrificed and then the draining auricular lymph nodes were removed and pooled (for each individual mouse) on day 3. Lymph node cell suspensions were prepared by mechanical tissue disaggregation through a sterile stainless steel gauze. Single cell suspensions were used to determine lymph node proliferation by comparing the cell count between retinol and vehicle treatment groups. Positive reactions were defined as either significant ear swelling or significant increase in lymph node cell counts (Homey *et al.*, 1998).

The differentiation index (DI) was calculated as the relationship between the activation of the skin-draining lymph nodes (percent of maximum increase in lymph node cell count index) and the acute skin inflammation (percent of maximum increase in ear thickness). The maximum ear swelling (15 × 0.01 mm) and a maximal lymph node cell count index (5) were estimated as mean value from large series of previous experiments (n = 50) with either strong irritants (eliciting maximal ear swelling) or potent strong allergens. To define criteria for the differentiation between

the allergenic and irritant potential of test chemicals, the relative degree of lymph node activation was compared with the relative degree of skin inflammation. For mathematical reasons, the maximal increase in the lymph node cell count index (maximal lymph node cell count index − 1 = 4) was used to classify the relative degree of lymph node activation.

% of maximal increase in LN cell count index

$$= \frac{\text{LN index} - 1}{4} \times 100$$

$$\% \text{ of maximal ear swelling} = \frac{\text{ear swelling (mm)}}{15 \times 0.01 \text{ mm}} \times 100$$

Differentiation index (DI)

$$= \frac{\% \text{ of maximal increase in LN cell count index}}{\% \text{ of maximal ear swelling}}$$

The DI describes the relation between the activation of the local skin-draining lymph nodes and the skin inflammation at the site of topical treatment. A value of DI > 1 indicates a sensitizing reaction whereas 0 < DI < 1 indicates an irritant potential of the tested substances.

Measurement of UVA-induced matrix metalloproteinase-1 in skin fibroblasts. Human dermal fibroblasts from the foreskin were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 0.48 mg/m*l* glutamine, 100 IU/m*l* penicillin, 50 mg/m*l* streptomycin, and 10% fetal bovine serum. Cells between the fourth and seventh passage were used for the experiment. Skin fibroblasts were seeded in 48-well plates (10^4 cells/0.5 *l*/well). Confluent cultures of fibroblasts were irradiated with Ultraviolet (UV) A (15 J/cm^2) using a high-intensity UVA source (Dermlight cube 401 equipped with UVA filters, Uvatec, Inc., Sherman Oaks, CA, USA) through the thin layer of PBS in the tissue culture plate. After irradiation, fibroblasts were re-fed with 0.5 ml of DMEM without serum containing t-ROL (1 or 10 µM) and t-RA (1 or 10 µM), and incubated for 48 hr. Interstitial collagenase was measured with the MMP-1 human ELISA system (Amersham Pharmacia Biothch, UK), and inhibitory effects of t-ROL and t-RA were evaluated (Yoshioka *et al.*, 1987).

Immunohistological measurement of type-1 pN collagen synthesis in hairless mice. Female albino hairless mice (Skh:hr-1), 6~8 weeks old, were obtained from Charles River Laboratories (Wilmington, Mass., USA), grown to 26 weeks, and then used for experiment. Solutions of vehicle (DAE 433), t-ROL (0.1%), or t-RA (0.1%) were applied to the dorsal trunk with occlusion for 6 days. Immunohistological analysis of type I pN collagen was performed according to the methods described previously (Kligman *et al.*, 1996). Type I pN collagen was detected with mice monoclonal IgG1 antibody (Santa Cruz Biotechnology, CA, USA) raised against the aminoprepeptide region of human type I procollagen. The quantity and intensity of redness under the

epidermis were examined for index of procollagen increment, and graded 0 to 4 point by increment of redness (0, similar to Control; 1, slight; 2, mild; 3, moderate; 4, severe). The thickness of epidermis was also measured.

RESULTS

Primary skin irritation test of t-ROL and t-RA. Both 0.1% of t-ROL and t-RA induced mild skin irritation. But the irritation induced by t-ROL was weaker than that of t-RA (Table 1).

Skin sensitization of t-ROL using IMDS. Significant increases in lymph node weights ($p < 0.05$) and cell counts ($p < 0.05$) were calculated for all concentrations (0.1%, 1% and 2%) of t-ROL and 1% DNCB, compared with the vehicle control (Table 2). t-ROL caused a dose-dependent increase in both lymph node weight and cell counts. Following the skin-draining lymph node cell proliferation, skin

inflammation was determined by measuring ear swelling in mice (Fig. 1). Topical treatment of t-ROL induced marked ear swelling compared with the vehicle control in a dose-dependent manner. A marked ear swelling ($35.4 \pm 9.21 \times 10^{-2}$ mm) was obtained by treatment of 2% t-ROL, which, in turn, was accompanied by significantly increased lymph node cellularity (Table 2, Fig. 1). Also, 1% of DNCB induced marginal but significant ear swelling. However, the degree of ear swelling in DNCB treated group was smaller than that of t-ROL.

In Table 3, the results regarding local draining lymph node cell proliferation and skin inflammation exerted by t-ROL are summarized. To quantitatively distinguish irritation reaction from allergy, a DI was calculated as outlined in Methods. Topical treatment with the contact allergen DNCB (1%) induced significant increase in lymph node cell count (110.7% of maximal increase in lymph node cell count index), and only marginal but significant skin inflammation (52.2% of maximal ear swelling). The DI for DNCB that is well-known for allergen was revealed 2.12, which clearly indicated its contact sensitizing potential. The three doses (0.1, 1 or 2%) of t-ROL showed 52.7 to 115.5% of maximal increase in lymph node cell count index, and strong inflammatory reactions (71.1 to 236.0% of maximal ear swelling). The treatment of retinol (0.1, 1 or 2%) pro-

Table 1. Primary skin irritation potencies of t-ROL and t-RA

Compounds	Concentration	P.I.I.*	Decision**
t-ROL	0.1%	0.89	Mildly irritant
t-RA	0.1%	1.83	Mildly irritant

*P.I.I., Primary irritation index = ∑Total Score of erythema/eschar (0 to 4) and edema (0 to 4)/(Animal No. × 4).
**Decision; 0~0.50, non irritant; 0.51~2.00, mildly irritant; 2.01~5.00, moderately irritant; 5.01~, severely irritant.

Table 2. Effects of t-ROL and t-RA on lymph node weights and cell counts

Test substances	Lymph node weight (mg)	Lymph node cell count ($\times 10^5$/ml)
Vehicle	2.52 ± 0.39	70.0 ± 18.05
0.1% t-ROL	4.03 ± 0.92*	217.5 ± 35.58*
1% t-ROL	6.01 ± 1.51*	361.7 ± 50.12*
2% t-ROL	6.88 ± 2.01*	393.4 ± 40.38*
1% DNCB	5.52 ± 1.34*	380.1 ± 35.12*

*, Significant difference from control group ($p < 0.05$).
Data were analyzed by using Student's unpaired t test

Fig. 1. Ear thickness following marginal skin inflammation induced by t-ROL or DNCB.

Table 3. Differentiation index (DI) of t-ROL or DNCB defined as the ratio of the percent increase in maximal LN cell count index to the percent of maximal ear swelling
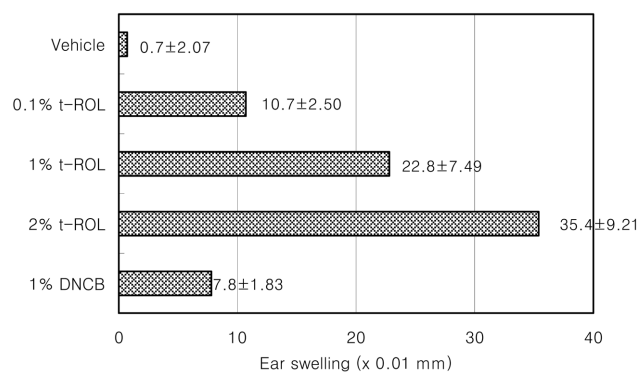
Test substances	LN cell count index		Ear swelling index		D.I.
	S.I.	% of maximal increase in LN	Ear swelling (0.01 mm)	% of maximal ear swelling	
Vehicle	1	-	0.7 ± 1.12	-	-
0.1% t-ROL	3.11	52.7	10.7 ± 1.07	71.1	0.74
1% t-ROL	5.17	104.2	22.8 ± 1.02	152.2	0.68
2% t-ROL	5.62	115.5	35.4 ± 1.13	236.0	0.49
1% DNCB	5.43	110.7	7.8 ± 1.08	52.2	2.12

S.I. (Stimulation Index), Ratio of lymphocyte proliferation treated test substances relative to vehicle-treated control.
D.I. (Differentiation index) = % of maximal increase (SI = 4) in LN cell count index/% of maximal ear swelling (15 × 0.01 mm).

Table 4. Inhibition rate of MMP-1 expression by t-ROL and t-RA after UVA-irradiation in human fibroblasts

Compounds	Rate of MMP-1 synthesis (%)*	
	1 μM	10 μM
t-ROL	90.8 ± 3.4	68.4 ± 5.9
t-RA	82.5 ± 6.3	10.2 ± 0.9

*These data showed inhibited MMP-1 synthesis by t-ROL and t-RA, when the MMP-1 synthesis by only UVA-irradiation without compounds was 100.

Table 5. Mean visualizing score (n = 8) of procollagen synthesis induced by t-ROL and t-RA

Compounds	Score (n = 8, $p < .05$)
t-ROL 0.1%	1.90 ± 0.61
t-RA 0.1%	2.95 ± 0.79

Grade; add 0 to 4 point by increment of redness (0, similar to Control; 1, slight; 2, mild; 3, moderate; 4, severe).

duced dose-dependent DIs ranging from 0.74 to 0.49, implying strong contact irritation potency.

Effect of t-ROL and t-RA on UVA-induced MMP-1. Any meaning effect was not detected at low concentration (1 μM) of both t-ROL and t-RA. However, high concentration (10 μM) of t-ROL and r-RA exhibited high inhibition rates, resulting in 30% and almost 90%, respectively (Table 4).

Procollagen synthesis in hairless mice. Procollagen synthesis was increased by treatment of t-ROL and t-RA (Table 5, Fig. 2). In comparison, t-RA exerted a higher potency in induction of procollagen synthesis than that of t-ROL. The thickness of epidermis remarkably increased ($p < 0.05$) following treatment with t-ROL (1.97 fold) and t-RA (2.56 fold), which is due to hyperkeratosis and acantosis (Table 6, Fig. 2). A characteristic of hairless mouse skin is the presence of horn-containing cysts in the lower dermis, which derive from embryonic hair follicles (Fig. 2) as

Table 6. Thickness (μm) of epidermis treated with t-ROL or t-RA (n = 8)

Compounds	Thickness (fold of control)
Vehicle (DAE 433)	21.6 ± 6.4
t-ROL 0.1%	42.5 ± 10.1 (1.97)*
t-RA 0.1%	55.2 ± 12.3 (2.56)*

*, Significant difference from control group ($p < 0.05$).
Data were analyzed by using Student's unpaired t test

reported previously (Mitani *et al.*, 2001).

DISCUSSION

Topical formulations of retinoids have been prescribed for various skin ailments without clinically significant side effects. But it was known that retinoids generally induced the local skin irritation (Kim *et al.*, 2003), although the natural retinol with activity similar to retinoic acid reduced the skin irritation in contrast with the irritating potential of retinoic acid (Fluhr *et al.*, 1999).

In this study, t-ROL and t-RA induced skin irritation in rabbits. It is difficult to estimate the allergic potential of retinoids using guinea pig maximization test (GPMT) which is usually used, because of skin irritation of retinoids. Therefore, we performed IMDS method that is an alternative to sensitization test to detect the allergic potential of t-ROL. Our results showed the t-ROL did not induce the allergy, and this IMDS is the good alternative method to supplement the limitations of GPMT.

The LLNA proposed by Basketter and Kimber (Basketter *et al.*, 1996; Kimber, 2001) greatly reduces time and cost to about 1/5 to 1/10 of those of GPMT test, and now is accepted as an OECD guideline through the validation studies (Basketter and Gerberick, 1996; Chamberlain and Basketter, 1996; Gamer *et al.*, 2008; Gerberick *et al.*, 2007; Kimber *et al.*, 1999). The LLNA is a method for the prospective identification of chemicals which have the potential to cause contact hypersensitivity. Sensitizing activity is
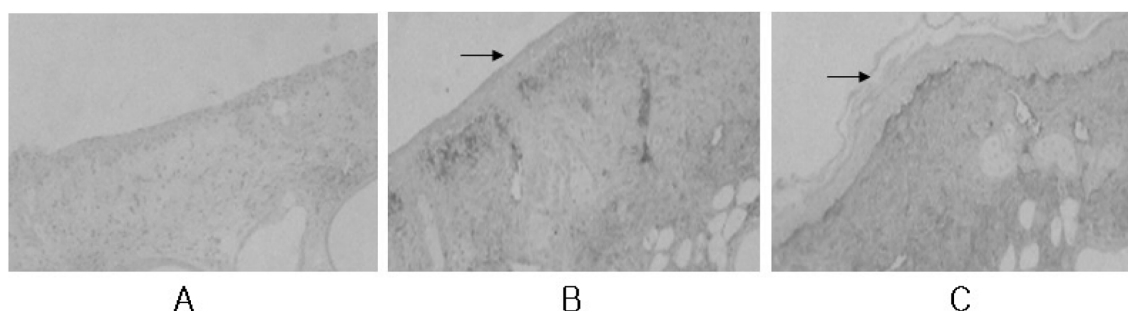
Fig. 2. Procollagen synthesis by vehicle (DAE 433) (A), 0.1% t-ROL (B) and 0.1% t-RA (C) in hairless mice (× 50). Procollagen is stained red under the epidermis. The thickness of epidermis was increased in t-ROL (1.97 fold) and t-RA (2.56 fold) treated groups because of hyperkeratosis and acantosis (arrows).

measured as a function of lymph node cell (LNC) proliferation induced in draining lymph nodes using ^{3}H-methyl thymidine following repeated topical exposure of mice to the test materials. The defect of this method is the using of radiolabeled isotope. Therefore many new approaches have been developed recently. A proliferative responses of LNC in rats (Arts *et al.*, 1996), mice (Jung *et al.*, 2010) and guinea pigs (Kashima *et al.*, 1996) were assessed by a non-radio-isotope method using bromodeoxyuridine (BrdU). Recently, cytokine measurement (Hariya *et al.*, 1999; Pichowski *et al.*, 2000; Ulrich *et al.*, 1998) or phenotype analysis using flow cytometry (Gerberick *et al.*, 1999; Jung *et al.*, 2010; Marnetz and Meade, 1999; Sikorski *et al.*, 1996) were performed to supplement the cell proliferation data. It is proposed that IMDS, used in this study, is considered to be a good method amending the defects of LLNA.

The anti-wrinkle effects of retinoids was also examined by estimating the UVA-induced MMP-1 and type-1 pN collagen synthesis. t-ROL and t-RA inhibited the UVA-induced MMP-1 in human skin fibroblasts and increased the type-1 pN collagen synthesis in hairless mice. The role of retinoids in the stimulation of collagen synthesis *in vivo* has been repeatedly demonstrated (Chen *et al.*, 1992; Griffiths *et al.*, 1993; Schwartz *et al.*, 1991; Wang *et al.*, 2002). Evidence suggests that this increase occurs via at least two mechanisms. One is the increment of steady-state levels of mRNA for types I and III procollagens (Schwartz *et al.*, 1991), and the other is inhibition of collagenase by retinoid-enhanced fibroblast secretion of tissue inhibitors of metall-proteinases, such as MMP-1 inhibitors (Clark *et al.*, 1987; Wright *et al.*, 1991). In this study, the results indicated that retinoids could reduce the wrinkle of skin by inhibiting the MMP-1 as well as by increasing the procollagen, when used in cosmetic formulation.

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