

The Effects of Retinoids on CRABP II mRNA Induction and Collagen Synthesis in Human Dermal Fibroblast.

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Retinoids are essential regulators of epithelial cell growth and cellular differentiation. They are also known to be effective in photo-aging. It was reported that topical application of retinoic acid improves facial wrinkle caused by collagen synthesis reduction in photodamaged skin. Collagen synthesis by retinoic acid may contribute to the wrinkle effacement. Since cellular retinoic acid binding protein II (CRABP II) is selectively induced in human skin and dermal fibroblasts *in vitro* by retinoic acid, this response can be used to measure retinoids potency and activity. In order to know the activity of retinoids and their relations with collagen synthesis, we treated dermal fibroblasts with retinoids (all-*trans* retinoic acid, retinol, retinaldehyde, retinyl palmitate) for 48 hours at 10^{-6} - 10^{-7} M and measured CRABP II mRNA level by quantitative Northern blotting. We also measured the rate of collagen synthesis by retinoids using 3-dimensional dermal equivalent. CRABP II mRNA level was increased 3-fold by retinoic acid, 2.1-fold by retinol and 1.4-fold by retinaldehyde. Collagen synthesis was increased 34% by all-*trans* retinoic acid, 26% by retinol, 17% by retinaldehyde and 7% by retinyl palmitate. From the above results, retinoids were found to be a potent inducers of CRABP II mRNA and collagen synthesis. Though retinoic acid was the most effective, its use has been restricted because of the side effects. Instead, retinol can be a best candidate in cosmetics for the treatment of photodamaged skin in terms of efficacy and safety.

Introduction

Topical application of all-*trans* retinoic acid(RA) has been shown to enhance repair of human and mouse skin damaged by chronic exposure to ultraviolet radiation(kligman et al, 1984; Bryce et al, 1988; Schwartz et al, 1991; Chen et al, 1992). Retinoic acid modulates the production of extracellular matrix components such as collagen, fibronectin and laminin(Varani et al, 1990)

The ability of topical RA to improve fine wrinkles associated with damage caused by exposure to sun light has known to associated with new collagen synthesis. Chen et al demonstrated that wrinkle effacement in RA treated mice with photodamage was correlated with increased collagen synthesis. The formation of collagen I was significantly decreased in photodamaged human skin and this process was restored by treatment with retinoic acid(Griffiths et al, 1993).

It was reported than CRABPII was selectively induced by RA in normal human dermal fibroblasts(Astrom et al, 1991; Elder et al, 1993) This response can be used to measure retinoids potency and bioactivity(Elder et al, 1996). In this study we examined the potency of retinoids on collagen synthesis and CRABPII induction by using *in vitro* cell culture and *in vivo* hairless mouse model to know whether retinol can be a coametrical ingredient which has a ability to improve photodamaged skin.

Materials and Methods

Chemicals All-*trans* retinoic acid, retinol(ROL), retinaldehyde(RAL) and retinyl palmitate(RPAL) were purchased from Sigma chemical co.(St. Louis, Mo). Retinoids were stored in small aliquots under liquid nitrogen dissolved in dimethylsulfoxide.

Cell Cultures Fibroblast cultures were initiated from biopsies of normal human skin. Tissue was minced and plated onto 75-T plastic tissue flasks. Cells were maintained in Dulbecco's modified Eagle's medium(DMEM) containing 0.48mg/ml glutamine and 100 IU/ml penicillin, 50mg/ml streptomycin, and 10% fetal bovine serum(FBS, Gibco BRL) at 37°C in a 5% CO₂ humidified atmosphere. Fibroblast cultures were subcultured by trypsinization. This study used between the fourth and seventh passages fibroblast.

Preparation of Three-Dimensional Dermal Equivalent System Three-dimensional dermal equivalents were prepared with a 24-well multi-chamber plate as based on the Coulomb B method(Coulomb et al, 1983) by mixing type I collagen gel, reconstituted buffer (2.2g NaHCO₃, 4.77g HEPES make up to 100ml 0.06N NaOH), and fibroblasts. The collagen matrix contracted during 1 day due to an active organization of collagen fibrils by the fibroblasts. After 1 day, three-dimensional dermal equivalents were maintained as described monolayer cell cultures.

Measurement for Synthesis of Collagen Cells inoculated onto 24-well multi-chamber plates were assayed at confluency. The culture medium was changed to Dulbecco's modified Eagle's medium(DMEM) supplemented with 10% fetal bovine serum(FBS) containing 20mCi/ml L[2,3,4,5-3H]-proline(NEN chemicals) and incubated at 37°C in 5% CO₂ incubator for 24hrs. The protein concentrations per well were assayed with the Protein assay kit(Bio-Rad). After labeling, proteinase inhibitors were added to the cultures. The media and the cells were mixed and then sonicated. Collagen synthesis was assayed by measuring the radioactivity of the media and cells together after limited degradation with purified bacterial collagenase, according to the method of Peterkofsky and Diegelmann(Peterkofsky et al, 1971). The relative rate of collagen synthesis to total protein synthesis was calculated with the assumption that collagen had an amino acid content 5.4 times higher than that of other proteins(Diegelmann et al, 1972). Collagen synthesis of three-dimensional dermal equivalents were measured in the same manner as the procedure above.

RNA Isolation and Analysis Total cellular RNA was extracted from fibroblast monolayers grown in 75-T plastic tissue flasks by a single-step method of RNA isolation(Chomczynski et al, 1987) using a commercially available acid guanidinium thiocyanate-phenol extraction reagent (Trizol reagent, Gibco BRL). 10mg aliquots of RNA were fractionated on a 1.0% agarose gel in 10mM phosphate buffer, pH 7, followed by capillary blotting onto a nylon membrane(Nytran, Schleicher & Schuell). Membranes were hybridized overnight at 42°C against cDNA probes labeled by random priming with digoxigenin-modified dUTP(Hdfke et al, 1992) in a DIG-

EASY Hyb^R solution(Genius system, Boehringer Mannheim, indianapolis, IN). After low- and high-stringency washes, signals were generated by incubation with an anti-digoxigenin antibody coupled to alkaline phosphatase and a chemiluminescent dioxetane substrate(Genius system, Boehringer Mannheim, Indianapolis, INC)(Hdfke et al, 1992). Hybridization signals detected on x-ray film were quantitated by densitometry and are reported relative to the expression of the “housekeeping gene” GAPDH(Arcari et al, 1984). The CRABP_{II} probe was random-primed from the 0.87-kb XhoI/PstI fragment of huCRABP_{II}-Astrom(Astrom et al, 1991).

Histology 8-10 week old female albino hairless mouse(Skh:HR-1) were obtained from Charles River Laboratories(Wilmington, MA). Retinoids were treated in EtOH/PG(70:30, v/v) with 0.6% for two weeks every day. Skin samples processed for hematoxylin and eosin were measured epidermal thickness at light micorscopy level.

Immunohistology Immunohistology of type I pN collagen was performed as Griffiths CEM’s method(Griffiths et al, 1992) to measeur the synthesis of new collagen synthesis. Type I pN collagen was detected with mice monoclonal IgG1 antibody (SP1.D8) raised against the aminopreptide region of human type I procollagen(Foellmer et al, 1983). Approximately diluted IgG1 was used as control for SP1.D8 antibody.

Results

CRABPII mRNA The results of Northern blot analysis of retinoids treatment are shown in figure 1 and summarized in figure 2. Retinoids were treated at 5×10^{-7} M each. A marked induction of CRABPII mRNA was noted in response to retinoic acid, retinol relative to control.(Fig. 2)

Collagen synthesis Collagen synthesis assay is summarized in figure 3. Collagen synthesis increased 34% by retinoic acid treatment, 26% by retinol and 17 % by retinaldehyde at 5×10^{-7} M each.

Histologic changes RA, ROL and RAL significantly increased epidermal thickness as compared with vehicle($23 \mu\text{m} \pm 4.2$). ($P < 0.01$, $n=5$)(Fig. 4)

Epidermal thickness was increased to $78 \mu\text{m} \pm 3.5$ by RA, $72 \mu\text{m} \pm 5.5$ by ROL , $68 \mu\text{m} \pm 2.9$ by RAL . There was no significant increase by retinyl palmitate($27 \mu\text{m} \pm 7.5$)

Collagen immunostaining Retinol treatment increased type I pN collagen immunostaining intensities in hairless mouse compared to vehicle(Fig. 5).

Discussion

Degradation of matrix protein such as collagen is thought to be responsible for the wrinkled appearance of photo-damaged skin(Kligman et al, 1986; Wlaschek. M et al, 1994). Recently, It was reported that retinoid acid can prevent photodamage(Fisher et al, 1996). Wrinkle effacement by retinoic acid was the result of new collagen synthesis in skin dermal part(Griffiths et

al, 1993).

Although retinoic acid has wide and strong effects in photodamaged skin, its use is restricted because of the side effects such as irritation and redness.

We studied the effects of retinoic acid, retinol, retinaldehyde and retinyl palmitate on CRABP_{II} mRNA induction. *CRABP_{II}* is a retinoids inducible gene and its induction can be used to measure the efficacy of retinoids(Elder et al, 1996). Among tested, retinol induced CRABP_{II} mRNA expression by 2.4-fold compared to control.

Collagen synthesis was increased 26% by retinol treatment in 3D-culture system(Fig. 3) and this results was correlated with in hairless mouse(Fig. 5). Though retinol was less potent than retinoic acid in CRABP_{II} induction and collagen synthesis, it has enough potency in our results and reported not irritable(Kang et al, 1995).

From above results, we think that retinol can be a good candidate for use in wrinkle effacement and prevention of photodamage in cosmetics.

Further study needed to know the *in vivo* effects(in human) of retinol in photodamage skin.

References

- Arcari P, Martinelli R, Salratore F : The complete sequence of a full length cDNA for human liver for multiple mRNA species. *Nucleic Acid Res* 12:9171 - 9189, 1984
- Astrom A, Tarakkol A, Petersson U, Cromie M, Elder JT, Voorhees JJ : Molecular cloning of two human cellular retinoic acid binding proteins(CRABP). Retinoic acid induced expression of CRABPII but not CRABPI in adult human skin in vivo and in skin fibroblast in vitro. *J Biol Chem* 266:17662-17666, 1991
- Bryce GF, Bogdan NJ, Brown CC: Retinoic acids promote the repair of the dermal damage and the effacement of wrinkles in the UVB-irradiated hairlessmouse. *J Invest Dermatol* 91 : 175-180, 1988
- Chen S, Kiss I, Tramposch KM : Effects of all-trans retinoic acid on UVB-irradiated and non-irradiated hairless mouse skin. *J Invest Dermatol* 98: 248-254, 1992
- Chomczynski P, Sacchi N : Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol- chloroform extraction. *Anal Biochem* 162: 156-159, 1987
- Coulomb B, Dubertret L, Bell E : Endogenous peroxidases in normal human dermis : a marker of fibroblast differentiation. *J Invest Dermal* 81: 75-78, 1983
- Diegelmann RF, Peterkofsky B : Collagen biosynthesis during connective tissue development in chick embryo. *Dev Biol* 28:443-453, 1972
- Elder JT, Cromie MA, Griffiths CE, Chambon P, Voorhees JJ: stimulus selective induction of CRABPII mRNA : A marker for retinoic acid

- action in human skin. *J Invest Dermatol* 100:356-359, 1993
- Elder JT, Kaplan A, Cormie MA, Kang S, Voorhees JJ: Retinoic acid induction of CRABP II mRNA in human dermal fibroblasts: use as a retinoid Bioassay *J Invest Dermatol* 106:517-521, 1996
- Fisher GJ, Datta SC, Talwar HS, Wang ZQ, Varani J, Kang S, Voorhees JJ: Molecular basis of sun-induced premature skin ageing and retinoid antagonism. *Nature* 379:335-339, 1996
- Foellmer HG, Kawahara K, Madri JA, Furthmayr H, Timpl R, Tuderman L : A monoclonal antibody specific for the aminoterminal cleavage site of procollagen type I. *Eur J Biochem* 134: 183-189, 1983
- Griffiths CEM, Russman AN, Majnudar G, Singer RS, Hamilton TA, Voorhees JJ : Restoration of collagen formation in photodamaged human skin by tretinoin. *N Engl J Med* 329:530-535, 1993
- Griffiths CEM, Russman AN, Majmudar G, Singer RS, Hamilton TA, Voorhees JJ : A photometric scale for the assessment of cutaneous photodamage. *Arch Dermatol Res* 128: 347-351, 1992
- Hdfke HJ, Sanger G, Kessler C, Schmits G : Sensitive hemiluminescent detection of digoxigenin-labeled nucleic acid protocol and its applications. *Biotechnology* 12: 104-113, 1992
- Kligman LH, Duo CH, Kligman AM: Topical retinoic acid enhances the repair of ultraviolet damaged dermal connective tissue. *Connet Tissue Res* 12:139-150, 1984
- Peterkofsky B, Diegelmann R : Use of a mixture of protease-free collagenase for the specific assay of radioactivity collagen in the

- presence of other proteins. *Biochemistry* 10:988-994, 1971
- Kang S, Duell EA, Fisher GJ, Data SC, Wang ZQ, Reddy AP, Tavakkol A, Yi JY, Griffiths CM, Elder JT, Voorhees JJ: Application of Retinol to human skin *in vivo* induces epidermal hyperplasia and cellular retinol binding proteins characteristics of retinoic acid but without measurable retinoic acid levels or irritation. *J Invest Dermatol* 105: 449-556,1995
- Schwartz E, Cruickshank FA, Mezick JA, Kligman LH: Topical all-trans retinoic acid stimulates collagen synthesis *in vivo*. *J Invest Dermatol* 96: 975-978, 1991
- Varani J, Mitra RS, Gibbs D, Phan SH, Dixit VM, Mitra R, Wang T, Siebert KJ, Nickoloff BJ, Voorhees JJ : All-trans retinoic acid stimulates growth and extracellular matrix production in growth-inhibited cultured human skin fibroblasts. *J Invest Dermatol* 94:717-723, 1990
- Griffiths CEM, Russman AN, Majmudar G, Singer RS, Hamilton TA, Voorhees JJ : A photonumeric scale for the assessment of cutaneous photodamage. *Arch Dermatol Res* 128: 347-351, 1992

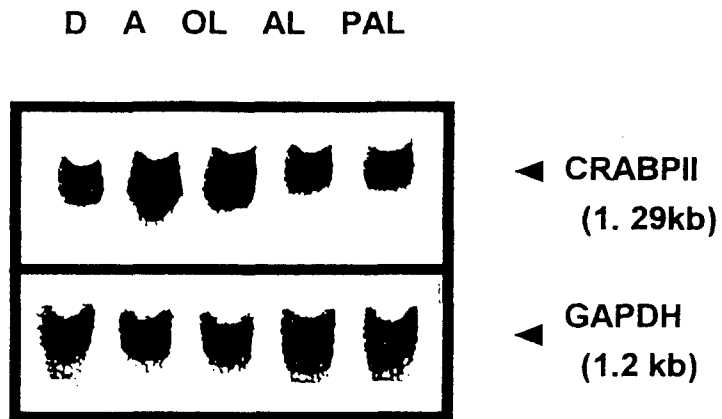


Figure 1. CRABP II mRNA is induced by retinoid in human dermal fibroblasts. Hybridizations of northern blots with CRABP II and GAPDH are shown. D ; DMSO, A; retinoic acid, OL; retinol, AL; retinaldehyde, PAL; retinyl palmitate

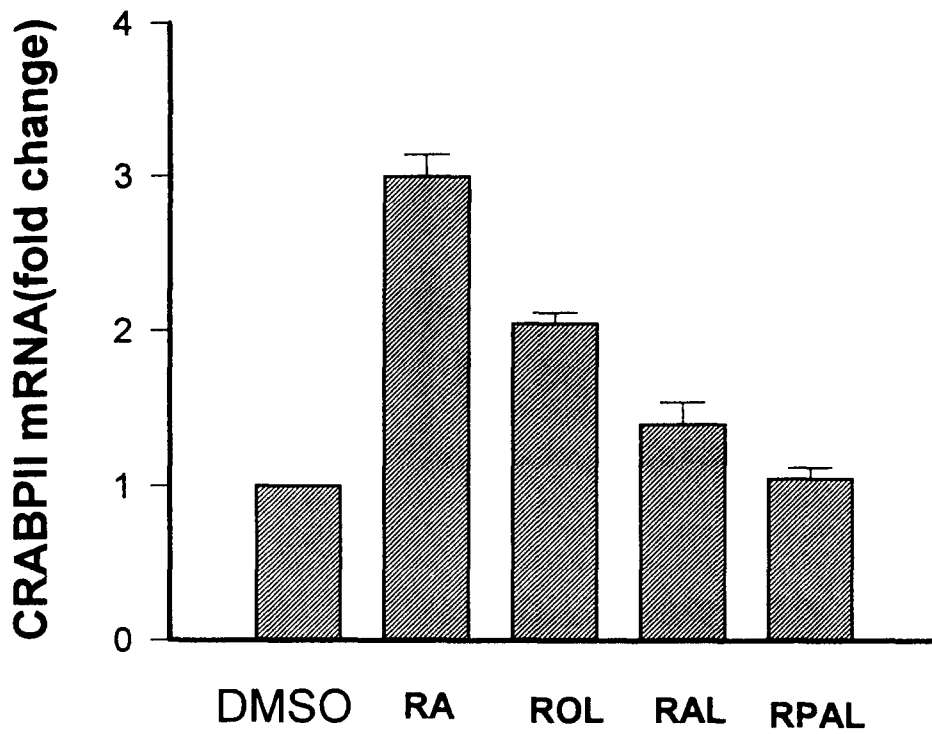


Figure 2. Summarized results of CRABP II mRNA induction in human fibroblasts. CRABP II is increased 3-fold by retinoic acid, 2.1-fold by retinol, 1.4 -fold by RAL.

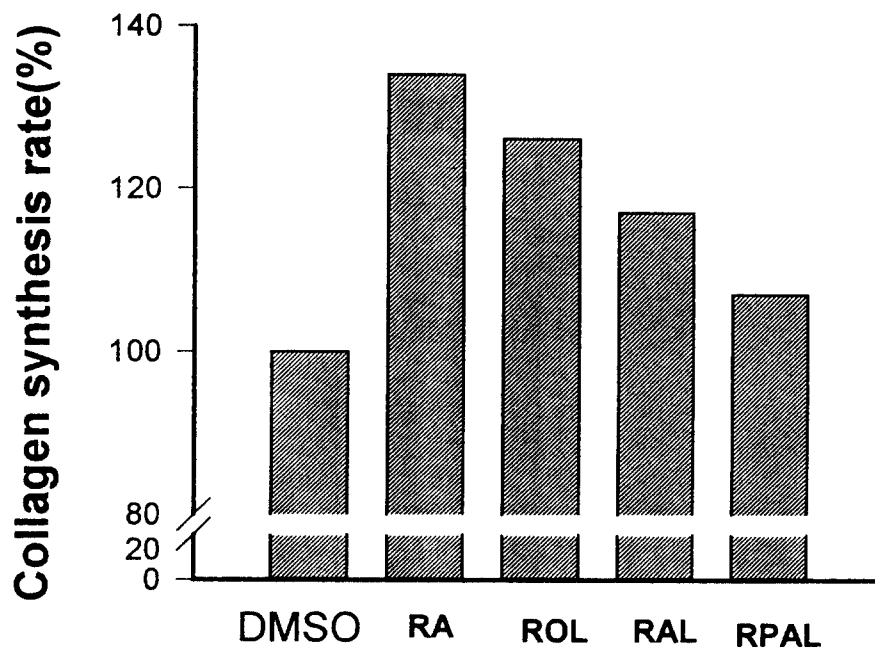


Figure 3. The effects of retinoids on Collagen synthesis.

Retinoids were treated at 5×10^{-7} M for 48 hours. Collagen synthesis was increased 34% by RA, 26% by ROL, 17% by RAL.

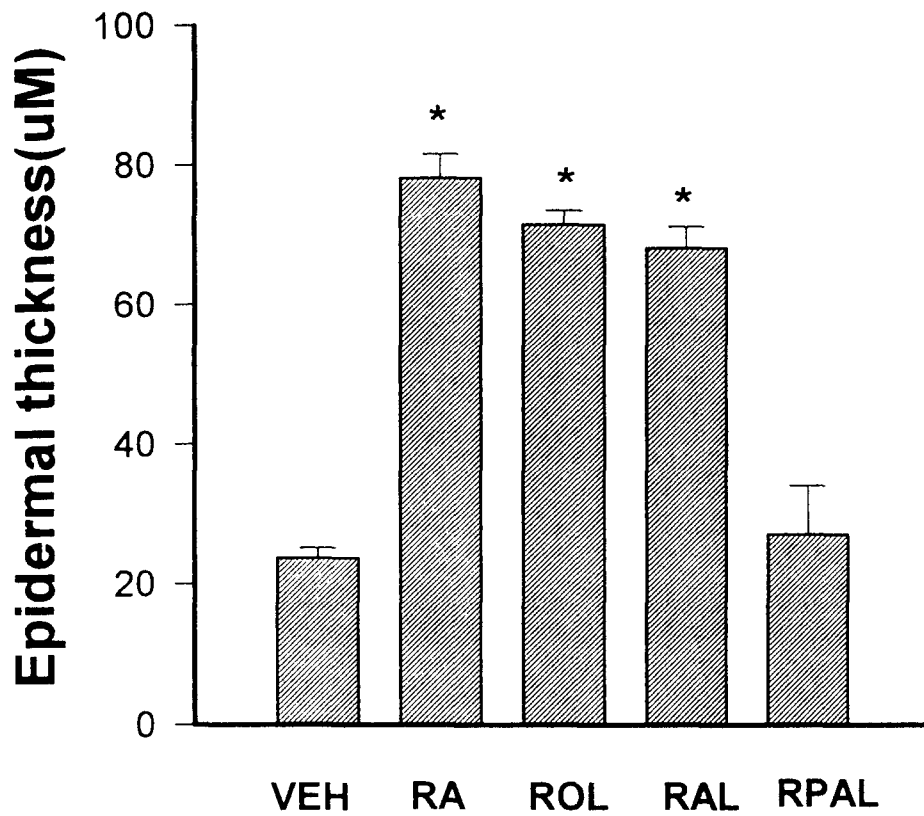
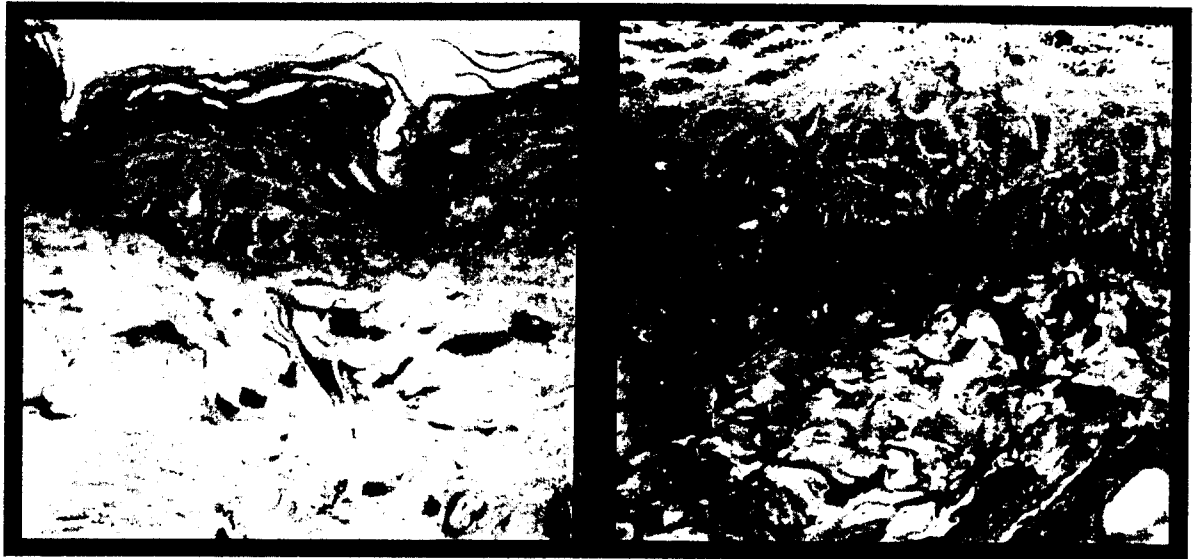


Figure 4. The Change of Epidermal thickness after topical application of retinoids.

(* P < 0.01 versus vehicle)



(A)

(B)

Figure 5. Collagen I immunostaining of hairless mouse skin(x 350). PN collagen I is stained with deeper red-color in the retinol treated(B) than vehicle treated(A).