# Topical Retinoic Acid Treatment of Photoaged Skin: Its Effects on Hyaluronan Distribution in Epidermis and on Hyaluronan and Retinoic Acid in Suction Blister Fluid

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Topical treatment with retinoic acid (tretinoin, vitamin A acid) has been reported to partly reverse signs of photodamage. To determine whether the histochemical distribution of hyaluronan (hyaluronic acid, HYA) in the epidermis and dermis and the amounts of HYA and retinoic acid in suction blister fluid were influenced by such topical treatment, 14 subjects healthy apart from moderate photodamage were instructed to treat the lateral forearm with 0.01–0.05% retinoic acid cream for 6 months. In a study of the short-term effects, another six subjects applied 0.05% retinoic acid cream for 2 weeks. After 6 months the thickness of the vital epidermis had increased by 23%. The HYA staining was based on a specific immunohistochemical method in which hyaluronan-binding protein is used. Before treatment HYA was seen as a meshwork around the cells in the upper half of the stratum spinosum. After 6 months of treatment this meshwork had increased in thickness by 31% compared with pretreatment specimens. The HYA staining was already intense in the papillary dermis before treatment and no difference was observed after 6 months' treatment. The mean concentration of HYA in blister fluid had increased significantly (43%) after 2 weeks of treatment whereas after 6 months there was no significant difference in this respect between the treated and untreated arm. The increase in the thickness of the epidermal HYA meshwork after 6 months and the blister fluid HYA after 2 weeks may indicate that HYA is involved in the epidermal change induced by topical retinoic acid therapy. The mean concentration of retinoic acid in suction blister fluid after 2 weeks of treatment was 328±63 nM whereas before treatment retinoic acid was usually not detectable. After 6 months of treatment the mean retinoic acid concentration was 73±33 nM. The mechanisms for the lower retinoic acid values at 6 months compared with those at 2 weeks are unknown. *Key words: Hyaluronic acid (HYA); HA-binding protein; Tretinoin; RA; Vitamin A acid; Retinol; Photoaging.*

Chronic exposure to sun leads to premature ageing of the skin, so called photoageing or dermatoheliosis. The sun-damaged skin is wrinkled, yellowish, lax, rough, dry, leathery, telangiectatic and spottily hyperpigmented. The main histological features of the photoageing include epidermal dysplasia with cytological atypia and loss of polarity of keratinocytes, dermal damage with marked elastosis, loss of collagen, an increase in glycosaminoglycans (GAGs) and modest inflammatory infiltrates (1). GAGs are part of the amorphous ground substance in the skin and are produced locally by fibroblasts (2) and keratinocytes (3). The predominating GAGs in the skin are hyaluronan (hyaluronic acid, HYA) and dermatan sulphate (4). Half of the body content of hyaluronan is located in the skin, mainly in the dermis (5), but there are also small amounts in the epidermis. Until recently, the physiological roles of HYA have been largely unknown.

High concentrations of HYA have been found during biological processes in which the extracellular matrix is remodelled, e.g. in the embryo during the morphogenetic phase and during early phases of wound healing (6, 7). HYA has been shown to influence cell differentiation, motility, proliferation and aggregation (8).

Vitamin A is necessary for normal differentiation of keratinocytes, and retinoic acid seems to be the biologically most active form of the natural retinoids in terms of control of epithelial differentiation (9). Topical treatment with retinoic acid has been reported to partly reverse signs of photodamage (1, 10, 11) and to reverse at least early actinic keratoses (12).

As it has been shown *in vitro* that retinoic acid may stimulate the synthesis of HYA (3, 13–15), it seemed of interest to investigate the possibility that a period of topical application of retinoic acid on human photoaged skin might have an effect on HYA in the epidermis and dermis.

## MATERIAL AND METHODS

*Healthy subjects treated with topical retinoic acid for 6 months*

Thirteen women and one man (39–60 years, mean 50.4) all healthy and not receiving any systemic or topical treatment, entered the study. All showed signs of mild to moderate photodamage of the skin on the lateral forearms and on the dorsa of the hands, such as fine wrinkles, roughness, dryness, hyperpigmented spots and decreased elasticity. None of the subjects had been exposed to the sun or had had any UV-light treatment during the last 2 months preceding the study.

Retinoic acid in a hydrophilic vehicle was applied once daily on the dorsal aspect of the left forearm and hand, preferably in the evening. Each subject was instructed to start with 0.01% retinoic acid cream for one month. If this concentration was tolerated, 0.025% retinoic acid cream was applied during the second month and finally 0.05% retinoic acid cream was used for four further months. The subjects were allowed to use emollient creams ad libitum. Exposure to sun or artificial irradiation for tanning purposes was not allowed and a sunscreen (SPF 15) was applied on sunny days.

*Healthy subjects treated with topical retinoic acid 0.05% for 2 weeks*

To determine whether short-term topical treatment with retinoic acid could have a different effect compared with a longer treatment period, another six subjects healthy apart from mild to moderate photodamaged skin were included in the study. One man and five women (40–53 years, mean 45.2) applied 0.05% retinoic acid cream on the dorsal aspect of the left forearm once a day for 2 weeks. None of the subjects were receiving any oral or topical medical treatment or had been exposed to UV irradiation during the last 2 months.

Table I. *The measurements of the tissue thickness were performed by two independent observers, A and B*

The percentage values refer to the mean of the changes in thickness after treatment.

| Observer | Increase in thickness of the epidermis | | Increase in thickness of the HYA-stained layers in stratum spinosum | |
|---|---|---|---|---|
| | % | p value | % | p value |
| A | 23 | 0.018 | 31 | 0.019 |
| B | 20 | 0.010 | 31 | 0.009 |

### Skin biopsies

Punch biopsy specimens were obtained from the lateral aspects of the arm before the start and at the end of the treatment period. The second biopsy was performed within 2 cm of the baseline biopsy. The specimens were fixed in 4% neutral buffered formaldehyde containing 1% cetylpyridinium chloride and embedded in paraffin. A series of 4 μm thick consecutive sections were stained for HYA (see below).

### Immunohistochemical staining for HYA

The staining procedure for HYA has recently been described (16). In this specific HYA method, biotinylated hyaluronan binding protein (HABP) was used (17).

The preparation of HABP has been described previously (17). The HABP was separated from the link proteins by means of a Sepharose-6B chromatography column (Pharmacia, Sweden). The purified HABP was biotinylated after aggregation with HYA in order to protect the binding site. Finally, the HABP-biotin was affinity-purified on an HYA-Sepharose column, dialysed, and stored at −20°C.

After deparaffinization, the slides were incubated with 1% bovine serum albumin to block non-specific binding sites. The slides were then washed and incubated with 3% $H_2O_2$ in the dark to destroy any endogenous peroxidase activity. After rewashing, the slides were incubated overnight with biotinylated HABP. This was followed by a new wash and incubation with Vectastain-Elite avidin-biotin complex (ABC) reagent (Vector Labora-tories, Burlingame, CA, USA) and diaminobenzidine tetrahydrochloride (Sigma). Finally, the slides were washed and coverslipped. For control of the binding specifity of the probe, one section from each biopsy was digested with 50–100 units/ml Streptomyces hyaluronidase (Seikagaku Kogyo Co.,Tokyo) for 4 h at 37°C and then treated as described above.

On each biopsy specimen three measurements of the thickness of the viable epidermis (excluding the stratum corneum) and the meshwork of HYA in the stratum spinosum were performed by two independent observers (A and B). The same microscope equipped with two objectives with different enlargements was used. The measurement units were arbitrary and the changes in the epidermal thickness after treatment were expressed in per cent.

### HYA in blister fluid

After 6 months of treatment with retinoic acid, suction blisters were raised on the dorsal aspect of both forearms in 11 of the 14 subjects. In the group treated with retinoic acid for 2 weeks, suction blisters were raised on the treated and untreated arm at the end of the treatment period. In both groups 12–18 h had elapsed between the last application of retinoic acid and the induction of blisters.

The suction blisters were produced according to the method of Kiistala (18), with a suction power of 300 mm Hg, and the fluid was collected after 2–3 h and stored at −20°C. The blister fluid was diluted in assay buffer 1:5.

The radiometric assay for HYA developed by Pharmacia Diagnostics, Uppsala, Sweden was used (19). This test is based on the use of specific hyaluronic acid binding proteins, HABP, isolated from bovine cartilage. First the hyaluronic acid in the sample is allowed to bind ^{125}I-labelled HABP in solution for 60 min. The unbound ^{125}I-HABP is then quantitated by incubating the sample with HYA covalently coupled to Sepharose particles of small size and low density. The particles remain suspended during 45 min of incubation. Sep-

aration is achieved by centrifugation followed by decantation. The radioactivity bound to the particles is measured. It is inversely proportional to the concentration of HA in the sample.

### Retinoic acid in suction blisters

The concentration of retinoic acid in suction blister fluid was analyzed by HPLC (high pressure liquid chromatography) according to the method of Vahlquist (20) the Briefly, the samples (20–45 ul) underwent alkaline hydrolysis after the addition of an internal standard (Ro 10–1670). After extraction with hexane, evaporation, redissolution in ethanol, agitation with ultrasound and centrifugation, the solution was subjected to reversed phase HPLC on a Nucleosil PEAB-ODS column eluted with acetonitrile:water (14:86). The HPLC system consisted of a Beckman 110B pump in combination with an NLDC detector operating at 360 nm. The samples were analyzed in duplicate, and the detection limit was 5 ng/ml blister fluid.

### Statistics

All measurements of tissue thickness were performed by two independent observers (A and B) using two different objectives with different enlargements. Two observers were used to check the reproducibility of the measurements. The units are arbitrary and different for each set of data and therefore the two sets are non-compatible in a statistical sense. The numbers within the two sets are of different magnitudes with different variances and thus require individual analyses in order to assess the statistical significance of the treatment. The percentage change due to treatment in each set was computed for comparative and confirmative purposes (Table I).

From each biopsy specimen one to three valid measurements were obtained. The statistical evaluation was based upon a two-way analysis of variance model with the healthy subject as a random factor and time (before and after) as a fixed factor. This statistical method was used for all variables except for HYA in suction blister fluid in subjects treated 2 weeks with tretinoin, were data allowed a paired t-test.

The values for retinoic acid in suction blister fluid are expressed as mean ± SEM.

## RESULTS

### Clinical results

After 6 months 50% of the subjects showed visible changes in the skin of the treated arm compared with the untreated one. In these subjects the skin had become more shiny and the pigmentations less marked, with fewer visible brownish spots. There were no obvious differences in appearance between the treated and untreated arm after the short treatment period of 2 weeks.

### Histological results

*Epidermal thickness.* The thickness of the epidermis (excluding the stratum corneum) increased by about 20% after 6 months of retinoic acid treatment compared with the pretreatment values (Table I).
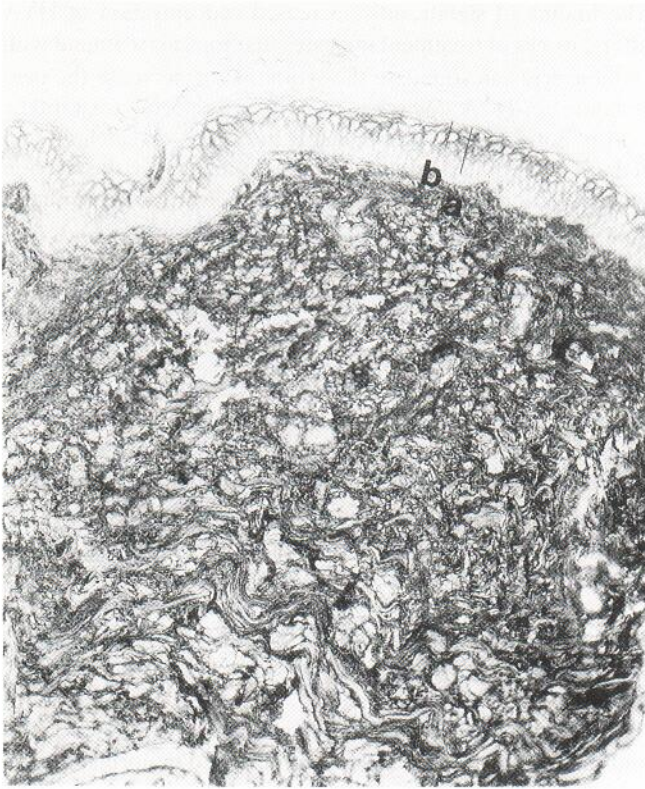
*Fig. 1.* A HYA-stained specimen from UV-exposed skin of the fore-arm before retinoic acid treatment. *a*) The thickness of the epidermis (stratum corneum not included). *b*) The thickness of the HYA-stained meshwork in the stratum spinosum.
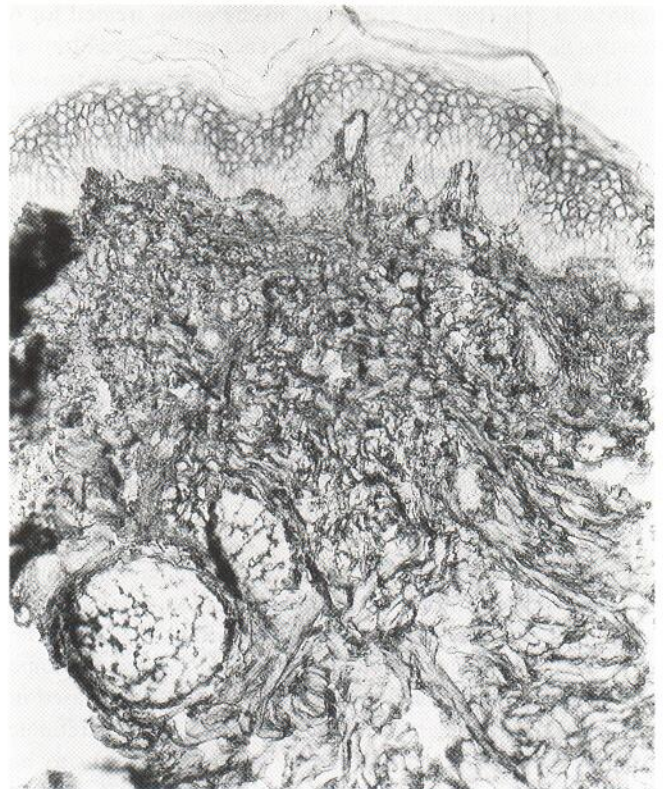


*Fig. 2.* HYA-stained specimens from the same person as in Fig. 1 after 6 months of retinoic acid treatment. The biopsy was performed within 2 cm of the baseline biopsy.

*HYA staining in the skin.* Before treatment, the HYA staining in the epidermis was concentrated to the intercellular spaces around the keratinocytes in the middle and upper part of the stratum spinosum, so that a meshwork was formed. In the basal layer the intercellular HYA staining was much weaker and in some specimens virtually no HYA was found around the lower parts of the basal cells. The stratum granulosum and stratum corneum were unstained. In the dermis, on the other hand, HYA was visible throughout the whole tissue. The up-per part of the papillary dermis, in particular, was intensely HYA-stained in all specimens. In the reticular dermis sparse staining of HYA was seen around the coarser collagen fibres and more marked staining was observed in the outer lining of the blood vessels. The same type of distribution of HYA in epidermis and dermis has been found in biopsies from UV-unexposed abdominal skin in healty subjects (unpublished data).

After 6 months of treatment the thickness of the HYA meshwork in the stratum spinosum was significantly increased (31%) (Table I, Figs. 1 and 2). The HYA staining in the stratum corneum and stratum granulosum remained negative. In the dermis no difference in the intensity of the HYA stain-ing was discerned after the 6-month treatment period com-pared with the pre-treatment observations.

*HYA in suction blister fluid.* The concentration of HYA in suction blister fluid was significantly higher (by 43%, $p=0.034$) in the forearm treated for 2 weeks than in the
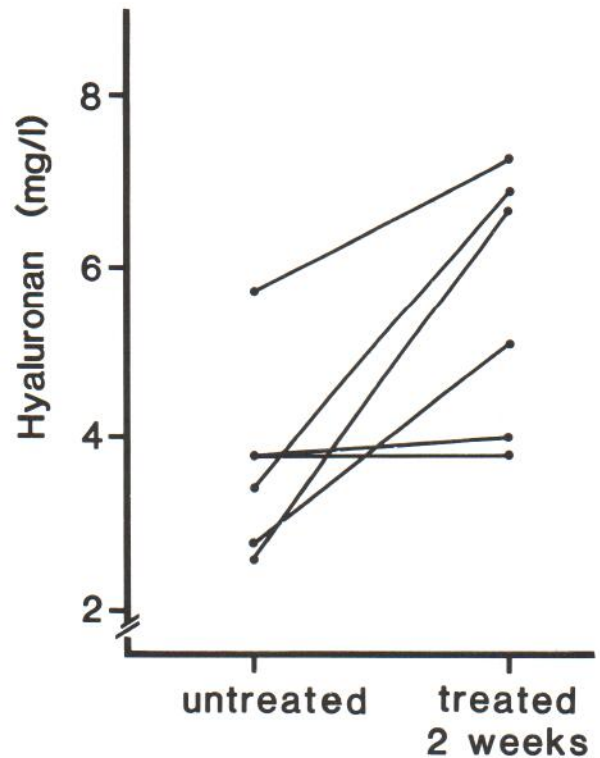


*Fig. 3.* The individual concentrations of HYA in suction blister fluid from the forearm treated with retinoic acid for 2 weeks and from the untreated arm. The lines connect dots representing the same person.

untreated arm (Fig. 3). However, in the group treated for 6 months there was no significant difference in the concentration of HYA in blister fluid between the untreated and treated arms. The concentration of HYA in suction blister fluid from untreated as well as treated arms were all within the previously described normal range found in healthy subjects in suction blisters from non exposed areas (21).

*Retinoic acid in blister fluid.* Retinoic acid was detected in blister fluid from 10/11 subjects after 6 months of treatment, 73±33 nM. Before treatment retinoic acid could be detected only in one subject (16 nM). In the 2-week treatment group all the 6 subjects demonstrated retinoic acid in blister fluid from the treated arm, 328±63 nM, but not from the untreated arm.

## DISCUSSION

The favourable effects of retinoic acid on human photoaged skin have been confirmed in several studies (10, 11, 22). Some of these effects were also observed in our study. Regarding the gross appearance, for example, the treated forearms became more shiny and the number of pigmented spots decreased in comparison with the untreated arms with use of 0.05% retinoic acid cream. The beneficial effects on the skin seem to be noticeable after 2–3 months whereas the irritating effects usually occur during the first month of treatment. Irritation was not experienced by all subjects, however, and the dorsal aspect of the forearm seems to tolerate the treatment better than the face, for example.

Histologically a 23% increase in the epidermal thickness (stratum corneum excluded) was observed after 6 months of treatment compared with the pretreatment specimens. This is in accordance with a previous report (9). It has recently been demonstrated that at least the same change in the degree of epidermal thickness as is induced by topical retinoic acid can be achieved by other local treatments, e.g. abrasive preparations (22).

The method used for histochemical localisation of HYA is both specific and sensitive (16). It is a modification of the method reported by Tammi et al. (23). Both in normal skin (23) and in untreated photoaged skin HYA was seen in the epidermis as a meshwork surrounding the keratinocytes in the upper half of the stratum spinosum, but it was not observed in the stratum granulosum or stratum corneum. The same type of distribution of the HYA meshwork in the upper part of the spinous layer was noted after 6 months of treatment. The thickness of the HYA epidermal meshwork increased by 31%. This is in line with the finding by Tammi et al. 1989 (15) that human epidermis cultured for 5 days with retinoic acid showed an accumulation of HYA particularly in the intercellular spaces of the spinous layers.

Since the immunohistochemical HYA method revealed no difference in the amount of HYA in the dermis after the 6-month treatment period compared with the pre-treatment specimens, we compared the HYA concentration in suction blister fluid from untreated and treated skin. Suction blister fluid is considered to represent dermal interstitial fluid (18).

The finding of significantly increased concentration of HYA after 2 weeks of treatment indicates that topical treatment with retinoic acid can stimulate the synthesis (or decrease the degradation) of HYA. Whether this effect is specific for retinoic acid is not known. Although the irritation, e.g. itching and redness, was mild or absent at the time when the suction blisters were induced, it cannot be excluded that the irritation in itself may lead to an increased production of HYA in a non-specific way. In fact, we have observed that very mild irritation, such as barely visible UVB-induced erythema, is associated with a transient increase in the concentration of suction blister HYA (unpublished data). After 6 months of treatment there was no significant difference in the HYA concentration in blister fluid between the treated and untreated arms. The concentrations were within the same range as previously has been reported in suction blisters from non-exposed skin in healthy subjects (21). This may possibly indicate that the long-term effects are not induced by irritation but by some other, more specific factor or factors.

The possibility of several causative mechanisms cannot be ruled out – one operative during the initial treatment period, when irritation is common and another, or others, when the irritation has subsided.

Retinoic acid is essential for epidermal cell differentiation and small amounts of it have been observed in normal human epidermis (20) and serum (24). We are not, however, aware of any reports on the presence of retinoic acid in suction blister fluid. The influence of ultraviolet radiation on the retinoic acid content has not been reported either. For retinol, the major retinoid of the skin, however, it has been shown that a marked but transient decrease takes place after exposure of the skin to ultraviolet radiation. This has been demonstrated in rabbit ear skin in vivo (25), in patients with uraemic pruritus, and in healthy controls undergoing UVB treatment (26). In the healthy persons the epidermal retinol concentration was reduced to a significantly subnormal level after 12 UVB exposures (8 J/cm$^2$) (26). The possibility of a UV-induced vitamin A deficiency in the skin which might enhance skin ageing and photocarcinogenesis has recently been discussed (27), but we are not aware of any controlled studies of epidermal retinoid levels in chronically sun-exposed skin.

Before treatment, retinoic acid in suction blister fluid could be detected only in one subject. After the short-term as well as the long-term treatment the mean concentrations were high (328±63 and 73±33 nM, respectively). The marked difference between the concentrations at 2 weeks and 6 months was, however, unexpected. In this context it is noteworthy that HYA in suction blister fluid was increased at 2 weeks but not at 6 months compared to before treatment. Factors that could influence the results might be lack of compliance (although this was denied by the participants), an induced degradation of retinoic acid during long-term treatment, or increased penetration in the early phase due to an inflammatory reaction and/or thinner epidermis than later. Another possibility is that the tretinoin treatment might influence the feed back loop regulating the synthesis of retinoic acid in epidermis (28).

The mechanisms of the beneficial effects of retinoic acid on sun-damaged skin are unknown. The results of recent *in vivo*

studies indicate that the nuclear retinoic acid gamma receptor might be the molecular target of retinoic acid in human skin (29). *In vitro*, retinoic acid has also been found to enhance both the stimulatory effect of epidermal growth factor and the inhibitory effect of transforming growth factor (30). Tammi et al. (15) reported that the incorporation of $^3$H-glucosamine into epidermal hyaluronate was stimulated by retinoic acid in human skin cultures. They suggested that this may be the cause of the delay in terminal differentiation and of the weakened cohesion of the keratinocytes induced by retinoic acid. Stimulation of epidermal synthesis of HYA both by retinoic acid and by other retinoids has been demonstrated in pig skin cultures (13). *In vitro* studies to compare the effects of retinoic acid with those of HYA on epidermal cell cultures are required in order to obtain more information about the role of HYA.

## REFERENCES

1. Weiss JS, Ellis CN, Headington JT, Tincoff T, Hamilton TA, Voorhees JJ. Topical tretinoin improves photoaged skin. A double-blind vehicle-controlled study. JAMA 1988; 259: 527–530.
2. Bashey RI, Perlish JS, Stephans RE, Fleichmajer R. Connective tissue synthesis by cultured scleroderma fibroblasts. II Incorporation of 3H-glucosamine and synthesis of glycosaminoglycans. Arthritis Rheum 1977; 20: 879–883.
3. Tammi R, Tammi M. Influence of retinoic acid on the ultrastructure and hyaluronic acid synthesis of adult human epidermis in whole skin organ culture. J Cell Physiol 1986; 126: 386–398.
4. Silbert J. Proteoglycans and glycosaminoglycans. In: Goldsmith L, ed. Biochemistry and Physiology of the Skin: Vol.1. New York and Oxford: Oxford University Press, 1983: 448–461.
5. Reed RK, Lilja K, Laurent TC. Hyaluronan in the rat with specific reference to the skin. Acta Physiol 1988; 134: 405–411.
6. Toole BP. Glycosaminoglycans in morphogenesis. In: Hay ED, ed. Cell Biology of Extracellular Matrix. New York: Plenum Press, 1981: 259–294.
7. Bently JP. Mucopolysaccharide synthesis in wound healing. In: Dunphy JE, Van Winkle W jr, eds. Repair and Regeneration. New York: Mc Graw-Hill, 1968: 151–160.
8. Toole BP, Munaim SI, Welles S, Knudson CB. Hyalronate-cell interactions and growth factor regulation of hyaluronate synthesis during limb development. In: Evered D, Whelan J, eds. The Biology of Hyaluronan. Chichester:John Wiley & Sons, 1989: 139–143.
9. Roberts AB, Sporn MB. Cellullar biology and chemistry of the retinoids. In: Sporn MB, Roberts AB, Goodman DS, eds. The Retinoids. New York: Academic Press Inc, 1984; 2: 209–286.
10. Kligman AH, Grove GL, Hirose R, Leyden JJ. Topical treatment for photoaged skin. J Am Acad Dermatol 1986; 15: 836–859.
11. Leyden JJ, Grove GG, Grove MJ, Thorne G, Lufrano L. Treatment of photodamaged skin with topical tretinoin. J Am Acad Dermatol 1989; 21: 638–644.
12. Peck GL. Topical tretinoin in actinic keratosis and basal cell carcinoma. J Am Dermatol 1986; Suppl 15: 829–835.
13. King IA. Increased epidermal hyaluronic acid synthesis caused by four retinoids. Br J Dermatol 1984; 110: 607–608.
14. Tammi R, Jansen CT, Tammi M. Effects of retinoic acid on adult human epidermis in whole skin culture. Arch Dermatol Res 1985; 277: 276–283.
15. Tammi R, Rípellino JA, Margolis RU, Maibach HI, Tammi M. Hyaluronate accumulation in human epidermis treated with retinoic acid in skin organ culture. J Invest Dermatol 1989; 92: 326–332.
16. Wells A, Larsson E, Tengblad A, Fellström B, Tufvesson G, Klareskog L, Laurent TC. The localization of hyaluronan in normal and rejected human kidneys. Transplantation 1990; 50: 240–243.
17. Tengblad A. Affinity chromatography on immobilized hyaluronate and its application to the isolation of hyaluronate binding from cartilage. Biochem Biophys Acta 1979; 578: 281–289.
18. Kiistala U. Suction blister device for preparation of viable epidermis from dermis. J Invest Dermatol 1968; 50: 129–137.
19. Brandt R, Hedlöf E, Åsman I, Bucht A, Tengblad A. A convenient radiometric assay for hyaluronan. Acta Otolaryngol (Stockh) 1987; Suppl 442: 31–35.
20. Vahlquist A. Vitamin A in human skin. I. Detection and identification of retinoids in normal epidermis. J Invest Dermatol 1982; 79: 89–93.
21. Lundin Å, Engström–Laurent A, Michaëlsson G, Tengblad A. High levels of hyaluronate in suction blister fluid from active psoriatic lesions. Br J Dermatol 1987; 116; 335–340.
22. Marks R, Hill S, Barton SP. The effects of an abrasive agent on normal skin and on photoaged skin in comparison with topical tretinoin. Br J Dermatol 1990; 123: 457–466.
23. Tammi R, Pipellino JA, Margolis RU, Tammi M. Localization of epidermal hyaluronic acid using the hyaluronate binding region of cartilage proteoglycan as a specific probe. J Invest Dermatol 1988; 90: 412–414.
24. De Leenbeer AP, Lambert WE, Claeys I. All–trans retinoic acid: Measurement of reference values in human serum by high performance liquid chromatography. J Lipid Res 1988; 23: 1362–1367.
25. Berne B, Nilsson M, Vahlquist A. UV irradiation and cutaneous vitamin A: An experimental study in rabbit and human skin. J Invest Dermatol 1984; 83; 401–404.
26. Berne B, Vahlquist A, Fischer T, Danielson BG, Berne C. UV treatment of uraemic pruritus reduces the vitamin A content of the skin. Eur J Clin Invest 1984; 14: 203–206.
27. Vahlquist A, Berne B. Sunlight, vitamin A and the skin. Editorial. Photodermatol 1986; 3: 203–205.
28. Duester G, Shean ML, McBride MS, Stewart MJ. Retinoic acid response element in human alcohol dehydrogenase gene ADH3: Implications for regulation of retinoic acid synthesis. Mol Cell Biol 1991; 1638–1646.
29. Elder JT, Fisher GJ, Zhang Q-Y, Eisen D, Krust A, Kastner P, Chambon P, Voorhees JJ. Retinoic acid receptor gene expression in human skin. J Invest Dermatol 1991; 96: 425–433.
30. Tong PS, Horowitz NN, Wheeler LA. Trans retinoic acid enhances the growth response of epidermal keratinocytes to epidermal growth factor and transforming growth factor beta. J Invest Dermatol 1990; 94: 126–131.