

## Light and Electron Microscopic Findings in Human Epidermis Reconstructed *In vitro* upon Topical Application of Liposomal Tretinoin

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**The topical application of tretinoin is a well-established approach to the treatment of acne vulgaris. However, induced inflammation, clinically addressed as a “flare-up”, is a major drawback. Recently, clinical and experimental investigations have hinted at a better tolerability, with equal efficacy, if the active compound is liposomally encapsulated.**

Using epidermis reconstructed *in vitro*, we compared the morphological changes upon topical application of a liposomal form (0.05% and 0.025%) and conventional form (0.05%) light and electron microscopically.

After 24 h several remarkable changes of the stratum corneum with all treatment modalities, representing inhibition of keratinisation wanted in acne vulgaris, were seen. When preparations of equal strength, i.e. 0.05%, were compared, the changes representing toxic dermatitis in the epidermis were more marked with the conventional form. Epidermis reconstructed *in vitro* treated with the liposomal forms showed no significant differences due to either concentration.

It is suggested that these changes correspond to the flare-up on clinical grounds. The *in vitro* findings further corroborate the hypothesis that liposomal encapsulation can increase the benefit/risk ratio of an active compound applied to the skin. **Key words:** retinoid; ultrastructure; human epidermis model.

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Tretinoin or vitamin A plays a major role in the proliferation and differentiation of epithelial structures. It is well-known from animal experiments that vitamin A deficiency leads to increased epidermal keratinisation. It was in fact this observation which early led to the topical use of vitamin A derivatives in skin diseases reflecting disturbed keratinisation such as psoriasis vulgaris, ichthyoses and acne vulgaris (1). The use of tretinoin in the topical treatment of ichthyoses, pityriasis rubra pilaris and actinic keratosis was first reported in 1962 by Stüttgen (2). Kligman et al. (3) were the first to demonstrate that topical application of tretinoin is a very efficacious mode of treatment for acne vulgaris, and that this effect is based on an increased production of non-coherent corneocytes in the follicle. Proliferation and retention hyperkeratosis tended to decrease, as is to be expected from the unusually stable coherence of corneocytes in these diseased states. Redness, scaling, itch and also temporary exacerbation of the acne itself (“flare-up”) were considered virtually unavoidable unwanted effects of this type of treatment.

Liposomal encapsulation in the past has repeatedly been proven useful to increase the benefit/risk ratio of an active compound destined for topical use. Thus liposomal encapsula-

tion of tretinoin might be an adequate galenic approach to decreased unwanted effects (4). In fact, in a half-sided double-blind clinical trial using liposomally encapsulated and free tretinoin in a conventional base commercially available, irritation was less marked with the former preparation while efficacy was alike in acne vulgaris (5). Changes of epidermal structures upon topical application of tretinoin, detected by light and electron microscopy, are well defined (6–15). In the following the morphological changes of human epidermis reconstructed *in vitro* upon application of liposomal and conventional topical tretinoin are investigated. The type of reconstructed human epidermis used here has been shown to be particularly apt for dermatopharmacological and dermatotoxicological investigations by Cannon et al. (16).

### MATERIALS AND METHODS

EpiDerm™ EPI-100 is a commercially available model of human epidermis (MatTek, Ashland, MA, USA). It consists of normal human keratinocytes cultivated in a serum-free medium (16). Human epidermis reconstructed *in vitro* was exposed to tretinoin in various forms, comprising a 0.05% conventional solution and liposomal forms of a concentration of 0.05% and 0.025%. Apart from the active compound the liposomes were composed of phospholipids and anti-oxydants. The liposomal dispersions containing tretinoin and the corresponding vehicle were a gift from Parfums Christian Dior, St. Jean de Braye, France.

Aliquots of 100 µl of the preparations were pipetted onto pieces of epidermal tissue of 0.6 cm<sup>2</sup>. The specimen proper as well as untreated controls were fixed in a cacodylate-buffered solution (0.05 M, pH 7.3) with 2.5% glutaraldehyde and 2.0% formaldehyde following standard methods (17). Postfixation of both parts was based on 1% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.3 at room temperature. After several washing and dehydration procedures (modified by starting with 70% ethanol in water with 1% p-phenylene diamine for 1 h at room temperature (18)) the Epon® embedding method was carried out. First semithin sections (1–2 µm) were studied with a light microscope after staining with 1% toluidine blue and 1% pyronine G. The small blocks of tissue were cut using an Ultracut ultramicrotome (Reichert, Wien, Austria). Ultrathin sections, 60 to 90 nm thick, were mounted on uncoated copper grids and stained in 2% uranyl acetate for 30 min, then in Reynold's lead citrate for 8 min, and examined using a Zeiss EM 902 transmission electron microscope (Zeiss, Oberkochen, Germany), operated at 80 kV, at magnifications ranging from ×3,000 to ×85,000.

### RESULTS

#### *Light and electron microscopic findings*

*Structure of untreated reconstructed epidermis.* The stratum corneum, composed of 20–25 layers of corneocytes, appeared



thickened, i.e. hyperkeratotic, and showed compact stacking. The individual cell layers of the rete Malpighii could be easily distinguished: upon the somewhat flattened basal cell lamina a normal squamous cell lamina and 2-4 strata representing the stratum granulosum could be seen. In particular, the border between stratum corneum and stratum granulosum could easily be detected. Only very rarely were there dyskeratotic cells. Keratohyalin granules appeared pleomorphic. Rete ridges were absent due to the artificial collagen bed used (Figs. 1a and 2).

The arrangement of intercellular lipids of the cornified envelope varied. In addition to typical multilamellar stacking (Figs. 3a, b and 4), there were irregularly stacked lipids (not

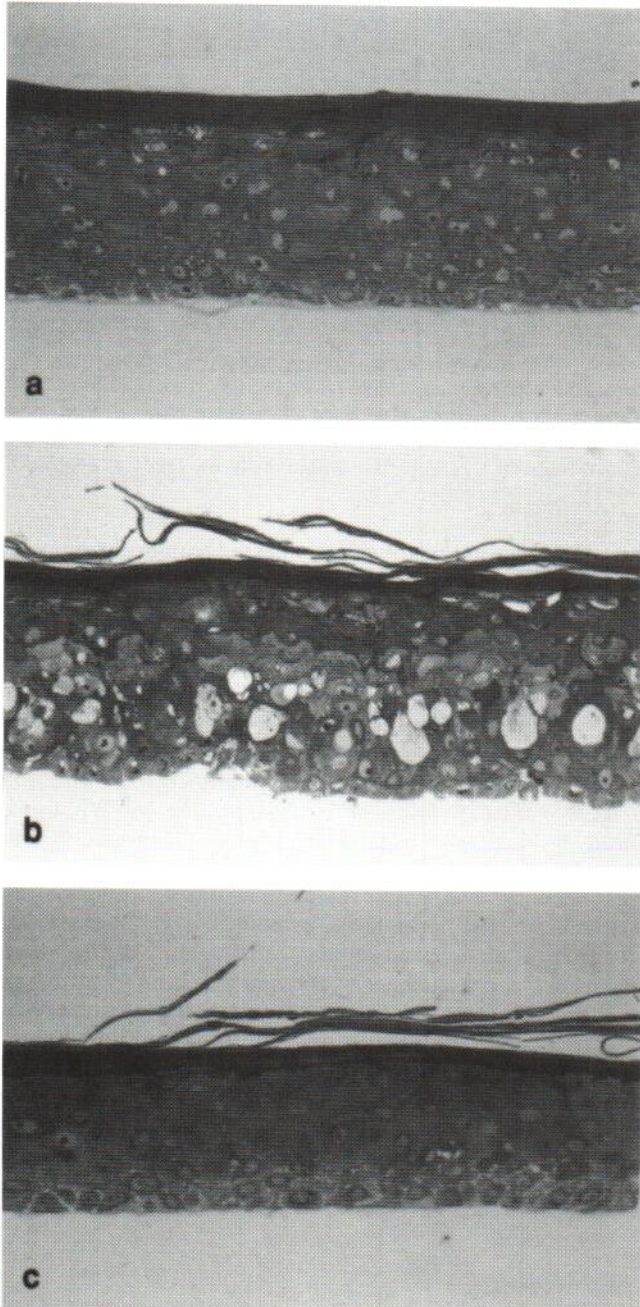


Fig. 1. Light microscopy  $\times 400$ . (a) Before treatment. (b) After treatment with 0.05% tretinoin for 24 h. (c) After treatment with 0.025% liposomal tretinoin for 24 h.

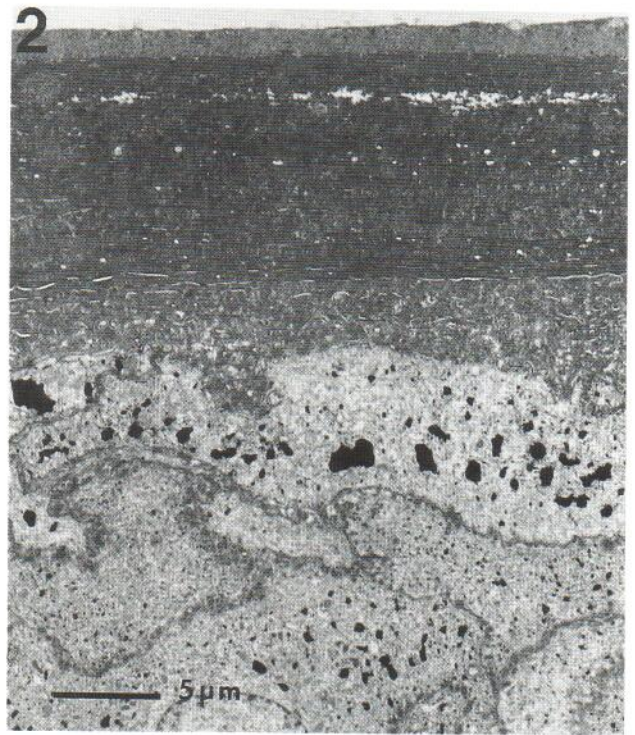


Fig. 2. Ultrastructural morphology of untreated reconstructed epidermis. Orthohyperkeratotic stratum corneum (20-25 layers). In vivo-like morphological characteristics of the rete Malpighii, for example showing keratohyalin granules within the stratum granulosum (2-4 layers) ( $\times 2,800$ ).

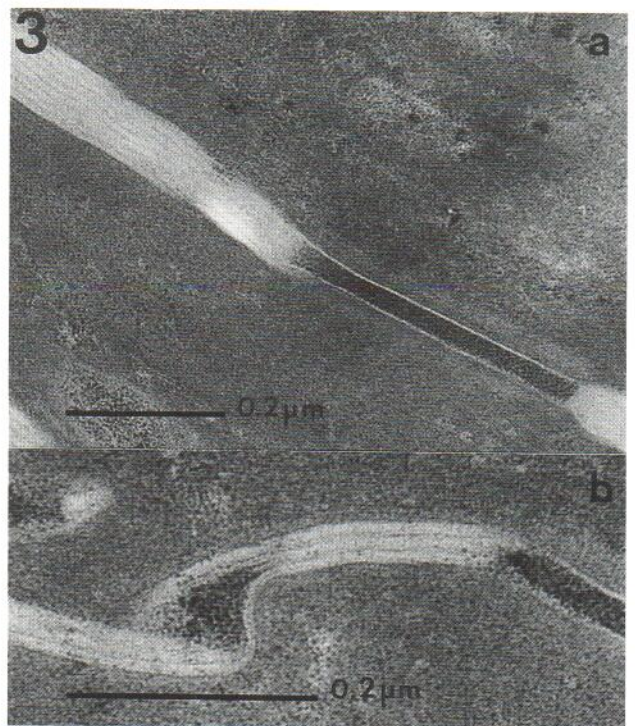


Fig. 3. (a-b) Ultrastructural morphology of untreated reconstructed epidermis. The lipid pattern in the intercellular space of the stratum corneum forms typical string-like arrangements. Notice the desmosomal plugs ((a)  $\times 104,000$ , (b)  $\times 165,000$ ).





Fig. 4. Ultrastructural morphology of untreated reconstructed epidermis. Forming of string-like lipid pattern (arrow) and amorphous lipid pattern (star) within saccular dilatation of the intercellular space at the stratum granulosum/stratum corneum interface ( $\times 72,800$ ).

shown) as well as material that appeared amorphous (Fig. 4). Numerous intercellular desmosomal connections were visible. In the only somewhat enlarged intercellular space between stratum granulosum and stratum corneum regularly stacked lamellar lipids and an amorphous lipid pattern could be detected (Fig. 4). Lamellar bodies were numerous and mostly unchanged. The well-known structures typical of normal skin, such as hemidesmosomes with anchoring filaments, subbasal dense plates, lamina lucida, lamina densa and anchoring fibrils, were seen.

*Structure of reconstructed epidermis after treatment with tretinoin solution 0.05%.* As compared to untreated skin, the usual layering of the epidermis with by and large equally sized and morphologically similar cells in the various strata was compromised. A marked decrease in the horny layer coherence of the thinned stratum corneum was seen, with clearly widened intercellular spaces. The stratum granulosum showed an enormous increase in thickness and partially deeply penetrated into the stratum spinosum, giving the image of a saw. Within all strata of living skin there were markedly swollen cells with pale cytoplasm, large perinuclearly situated vacuoles and dyskeratotic cells (Fig. 1b). At the ultrastructural level there was a loss of the desmosomal cell connections within the stratum corneum. The amount of lipid in the widened intercellular spaces being markedly decreased, a regular lamellar stacking was totally absent. The number of corneocyte layers was decreased as well, and the keratin pattern was also changed within the horny cells. Some of the corneocytes were less electron dense (Fig. 5). The stratum granulosum was thickened up to eight layers. Also at the ultrastructural level it could no longer be clearly distinguished from stratum corneum and

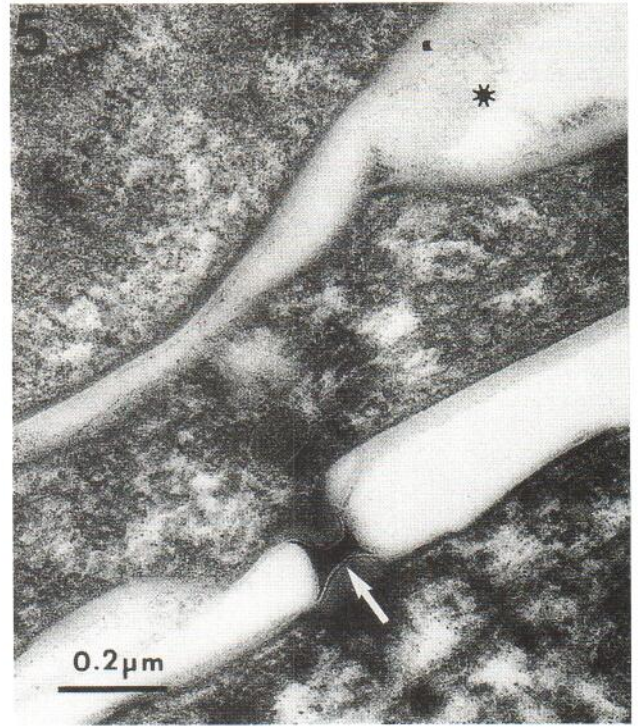


Fig. 5. Ultrastructural morphology of reconstructed epidermis after treatment with tretinoin 0.05%. Destruction of the desmosomal disks (arrow) within the irregular widened intercellular spaces of the stratum corneum. Complete loss of the typical intercellular lipid arrangement (star). Irregular keratin pattern within the horny cells ( $\times 72,600$ ).

stratum spinosum. Dark electron dense keratohyalin granules were visible (Fig. 6). Markedly damaged cells with pale cytoplasm and reduced number of desmosomes and tonofilaments were visible. Intracellularly multiple small vacuoles as well as giant – often perinuclearly situated – vacuoles could be found. With increase of oedema, the markedly swollen epidermal cells showed dissolution of cytoplasmic organelles. Other cells showed an increased number of swollen mitochondria (Fig. 6).

*Structure of reconstructed epidermis after treatment with 0.025% and 0.05% liposomal tretinoin preparation.* Within the horny layer compact hyperkeratosis was no longer to be seen, due to the widened intercellular spaces and the loss of desmosomal contacts. The keratin pattern was markedly altered, in particular in the upper layers of corneocytes. On the whole the changes observed correspond to the ones seen after treatment with a conventional tretinoin preparation (Fig. 1c). In contrast, however, the border between stratum granulosum and neighbouring strata was well defined. Keratohyalin granules appeared equally electron dense (Fig. 7), and intercellular lipids in the transitional area between stratum granulosum and stratum spinosum were stacked more regularly. Within the living epidermis morphology reflects the one with untreated controls. By and large, there were no clear-cut differences due to either concentration.

## DISCUSSION

Morphologic changes due to topical retinoid treatment have been described in several studies using animal models, organ cultures and human epidermis. However, in widely differing



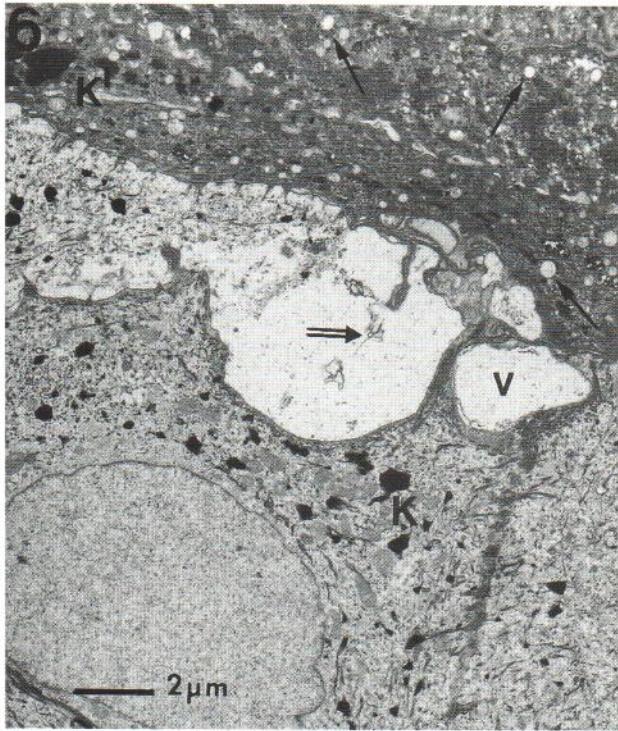


Fig. 6. Ultrastructural morphology of reconstructed epidermis after treatment with tretinoin 0.05%. Multiple vacuoles at the stratum corneum/stratum granulosum interface (arrows). Notice the different electron density of the keratohyalin granules (*K*, *K*) within the stratum granulosum. Markedly altered cell with vacuoles (*v*), pale cytoplasm and diminished number of tonofilaments and desmosomes. Membrane remnants of ruptured cell organelles (double arrow). Enlarged number of swollen mitochondria ( $\times 5,450$ ).

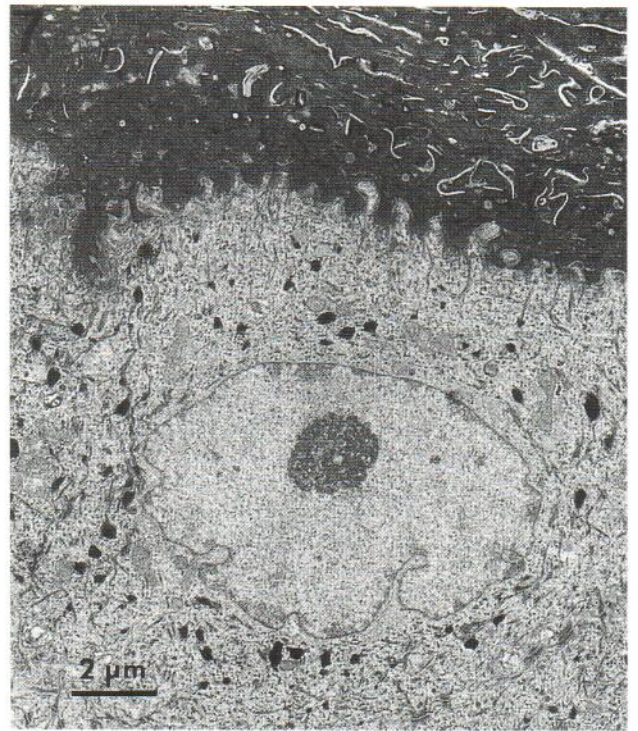


Fig. 7. Ultrastructural morphology of reconstructed epidermis after treatment with liposomal tretinoin 0.025%. Usual morphological structure of the stratum corneum/stratum granulosum/stratum spinosum interface ( $\times 5,450$ ).

assays the following changes were found: epidermal hyperplasia (8, 9, 11, 13), intercellular and intracellular oedema (7, 10, 12, 15), decrease of the number of tonofilaments (6, 7, 9, 12, 14, 15), increase of the intracytoplasmic glycogen content, polysomes and ribosomes (6, 7, 9, 12), presence of necrotic cells (7), reduction of desmosomal contacts with consecutive disorganisation and loss of cohesion of the stratum corneum (7, 9, 10, 12, 14, 15), accumulation of intra- and intercellular amorphous material and increase of the mitotic rate (8, 10, 12, 14, 15). With respect to changes of the stratum granulosum hyper- as well as hypogranulosis were observed (11–15). On the whole, the changes found were interpreted as an anti-keratinisation effect (8, 9). This effect seems to be therapeutically rewarding in the treatment of acne vulgaris, with hyperkeratosis representing a major aetiopathogenetic factor. A well-known, unwanted effect of this so-called exfoliating therapy is sometimes marked irritation of the skin. To decrease the unwanted effect while maintaining identical efficacy the use of lower concentrations of active compound in liposomal formulation seems to be helpful. It is this reduction of unwanted effects with concomitant increase of desired effects which has been postulated to be a consequence of liposomal encapsulation (4). Both in experimental and clinical trials liposomally encapsulated tretinoin was considered superior compared to the conventional preparation (5, 19, 20). In particular a reduction of irritation of the skin upon the use of the liposomal preparation was recorded (5, 21).

To verify the hypothesis of an increased benefit/risk ratio

we used a model of epidermis reconstructed in vitro which had proven to behave as an equivalent to human skin. Both at the light and electron microscope level all characteristics of living epidermis were seen, as was hyperkeratosis, a well-known phenomenon with such a type of skin, representing one of the relevant pathogenetic factors in the development of acne vulgaris. The epidermis equivalent used here shows a marked mitotic and metabolic activity. Moreover several epidermis-specific markers of differentiation such as pro-flaggrin and involucrin have been demonstrated (16). In lipid analysis and diffusion chamber experiments a by and large intact barrier function of the cornified envelope was shown, which is a pre-requisite for the performance of pharmacological experiments (16).

With all three types of preparations tested there were marked changes of the stratum corneum. However, the differences between the three sets of experiments were only minor. The apparent keratinisation effect of tretinoin could be corroborated using reconstructed epidermis, as was done by several investigators using differing types of skin (6–15). In our experiments, however, the above described results were seen even after 24 h, while other authors described corresponding morphological alterations only after several days of tretinoin treatment (6–8, 10, 12, 14, 18). Reconstructed human epidermis used here seems to be a very sensitive skin model for pharmacological experiments. The change in the structure of the cornified envelope is considered helpful under therapeutic conditions to reduce hyperkeratosis, which is a major factor in the development of comedonal acne. Reduced electron density of the superficial corneocytes was observed after treatment with tretinoin. This disturbance of the normal keratin



pattern might be a result of the keratolytic activity of tretinoin. A possible advantage of the use of liposomally encapsulated active compounds for topical use seems to be an increased formation of a depot within the stratum corneum (4). This might explain the about equal effect of 0.025% and 0.05% tretinoin on the stratum corneum. In fact, in the animal model a 5–10 times less concentrated preparation of the liposomal type was shown to have the same comedolytic activity as a conventional preparation of usual strength (21).

While changes in the stratum corneum were roughly the same with all three preparations, there were clear-cut differences with respect to living epidermis. On the whole the signs of toxic dermatitis were markedly less obvious upon treatment with liposomal tretinoin. It is these changes of living epidermis which seem to correlate to unwanted clinical effects, such as erythema and scaling, upon the application of tretinoin not liposomally encapsulated. Thus liposomal encapsulation can reduce this unwanted effect markedly. While there were already hints from clinical studies that liposomal tretinoin is at least as efficacious in the treatment of acne and better tolerated this could first be demonstrated on the basis of cell culture experiments. Thus the present results also contribute to the opinion that experiments based on human reconstructed epidermis in vitro can serve to reduce pharmacological experiments in man and animals.

However, it must not be forgotten that the barrier function in the case of reconstructed human epidermis is probably still different from that of normal human skin. In fact, there are still differences both in terms of structure and function. Essential lipid components seem to be differently arranged or even different by nature, and transepidermal water loss still is exceedingly high (22). These differences may well influence the relative uptake of free and encapsulated active compounds, such as tretinoin, and thus their relative activity.

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