

## Azelaic Acid vs. Placebo: Effects on Normal Human Keratinocytes and Melanocytes

### *Electron Microscopic Evaluation after Long-term Application in vivo*

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The effects of topically applied 20% azelaic acid (AA) on normal human epidermis were investigated vs. placebo in a double blind study by electron microscopy in 15 volunteers. After 3 months of local application twice daily, the pattern of epidermal keratinization was found altered in skin treated with AA. In particular, the number and thickness of tonofilament bundles and the number of keratohyaline granules seemed decreased; the remaining granules were smaller, occasionally showing irregular electron densities. The perinuclear endoplasmic reticulum and the cytoplasmic cisternae were enlarged and swollen mitochondria were regularly observed in most malpighian keratinocytes. Thorough quantitative evaluation of the number and distribution of melanocytes by a MOP-videoplan computer system showed no differences between verum and placebo sites, although, the mean number of melanocytes had increased in both, as compared to the untreated controls taken before onset of therapy. No significant qualitative changes of the normal melanocytes were found. These findings indicate that azelaic acid may influence the differentiation of normal human keratinocytes by reducing the synthesis of keratin precursors and may, therefore, act as a mild antikeratinizing agent, whereas, the pigmentary system in normal human epidermis does not show any specific change after 3 months of treatment with AA. (Received August 10, 1986.)

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Azelaic acid (AA) is a saturated dicarboxylic acid that has been reported to act beneficially on papulopustular and conglobate acne when topically applied in a 15% cream preparation (1). *In vivo* and *in vitro* experiments suggest that AA is a competitive inhibitor of some mitochondrial oxido-reductases (2, 3). The drug may possess bacteriostatic properties on aerobic and anaerobic bacteria, including *Propionibacterium acnes* (4, 5) and was suggested to exert a competitive inhibitory effect on the conversion of testosterone to 5-dehydro-testosterone (1, 5, 6).

These effects and, in addition, a possible interference with sebum production were thought to be related to the clinical improvement after topical AA application in patients with acne, but the exact mechanism of AA in this disease still remains unknown. Recently, *in vivo* studies on human subjects revealed that topical application of AA as a 20% cream does not change sebum production in human skin quantitatively, neither has AA a considerable effect on its qualitative composition (7).

The aim of the present investigation was to elucidate the *in vivo* influence of topically applied AA on normal human epidermal keratinocytes and melanocytes.

### MATERIAL AND METHODS

A randomised, double blind clinical trial had been designed to study the effects of AA on normal human skin. For this study 15 healthy volunteers had applied topically, twice daily over a period of 3

months, a base cream containing 20% AA on one shoulder (verum site) and the base cream alone on the other (placebo site). These individuals were not allowed to be exposed to acute U V radiation during the time period of the study or to be submitted to any other topical or systemic treatment. For evaluation, 3 skin biopsies were obtained in each of the 15 volunteers: one before any application (control biopsy) and two after 3 months of local application, one from the verum and one from the placebo site.

For the electron microscopic study a total number of 45 specimens were evaluated: 15 pre-treatment control biopsies, 15 after-treatment biopsies from the placebo site and 15 after-treatment biopsies from the verum site.

The specimens were prefixed in a freshly prepared solution consisting of 2% paraformaldehyde, 1.25% glutaraldehyde and 0.05%  $\text{CaCl}_2$ , buffered with 0.1 M Na-cacodylate buffer at pH 7.3, for 2 h at room temperature. The specimens were then minced with razor blades, and the fixation was continued for 12–24 h at 4°C. After brief rinsing in cold buffer, the specimens were postfixated for 1 h in 2%  $\text{OsO}_4$  solution in Na-cacodylate buffer, dehydrated in rising concentrations of ethanol and embedded in EPON 812, via propylene oxide. Thin sections were stained with uranyl acetate and lead citrate and examined at 60 KV with a Zeiss 10C electron microscope. Semithin 0.5  $\mu\text{m}$  sections were also made from the same blocks and were stained with toluidine blue for light microscopical observation. Both light and electron microscopic studies were performed in a blind fashion.

For additional quantitative evaluation of the number and the distribution of epidermal melanocytes, a part of each specimen was fixed in 4% formaldehyde buffered at pH 7.4 for 24 h, embedded in paraffin and 5–7  $\mu\text{m}$  sections were stained with HE and with Fontana-Masson's silver impregnation method.

The sections were then digitated via a digitizing board to a MOP-videoplan (Kontron) computer system connected with a Polyvar Reichert-Jung microscope. A standard measurement program had been used to count numbers of melanocytes as related to the measured lengths of basement membranes.

The number of melanocytes per mm of basement membranes was first calculated for each specimen and was then evaluated with statistical analysis for every individual and for each group of specimens from the verum and the placebo sites. Student's *t*-test was used to obtain the *p* values.

## RESULTS

### *Clinical follow-up*

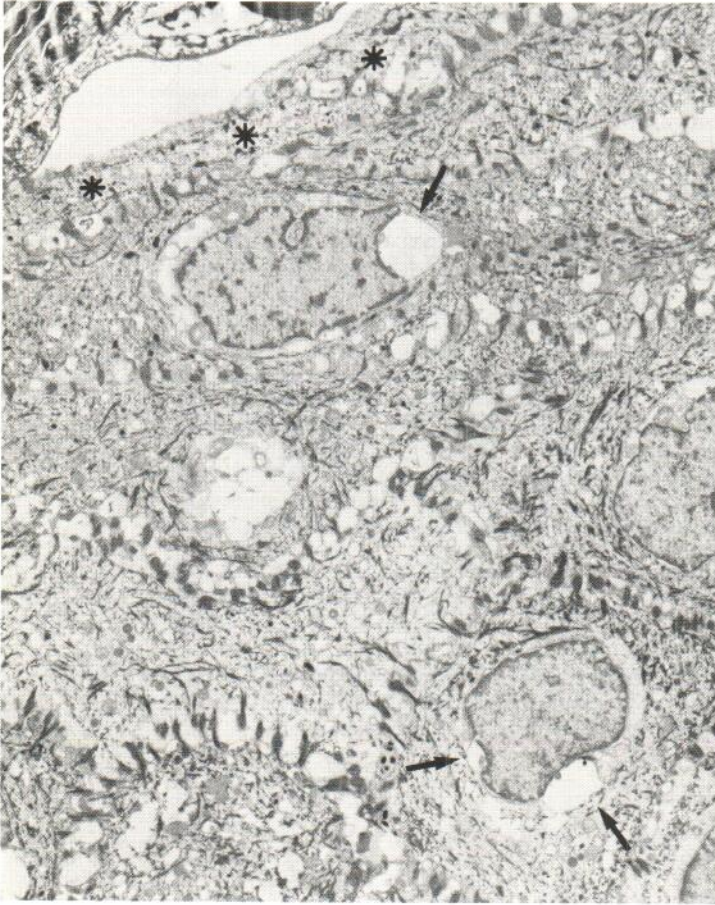
After 3 months of treatment no significant differences could be seen between the skin areas treated with AA compared to those treated with placebo. No hypo- or hyperpigmentation was observed on plain naked-eye observation. However, during the first weeks of AA application a discrete red erythema and/or a fine pityriasiform scaling were observed in most areas treated with AA. Moreover, during the first two weeks, some individuals referred prickling, burning and/or pruritic sensations, lasting 20–30 min. after each treatment at the verum site.

### *Electron microscopic evaluation*

*Controls.* In specimens from both the untreated controls and the placebo sites normal keratinocytes were found with the usual amount and distribution of cell organelles, tonofilaments and desmosomes. The number and size of the melanosomes varied greatly, but no abnormal changes of any type were observed. The pattern of keratinization was found normal in all specimens examined and well developed keratohyaline granules were present. Some melanocytes showed rarely swollen mitochondria, but their number remained within limits admitted as being acceptable in our laboratory. No intercellular oedema was seen. In general, no significant differences whatsoever could be detected between specimens from control biopsies and those from skin treated with placebo.

*AA-treated skin.* In contrast, discrete but distinct ultrastructural alterations were regularly observed in the epidermis of specimens from AA-treated skin. In several instances the mitochondria of keratinocytes were swollen, clearly showing loss of their cristae and





*Fig. 1.* Granular and upper malpighian layers of normal human epidermis treated with AA. The keratinocytes show irregular nuclei with dilated rough endoplasmic reticulum in the perinuclear area (arrows) and decreased amount of tonofilaments. At the granular layer only few and small keratohyaline granules are seen (stars). Intercellular oedema is also observed ( $\times 5\ 200$ ).

their matrix, despite showing a well preserved envelope. The number of tonofilaments seemed to be reduced and their bundles were thinner than usual. In the higher malpighian and granular layers, mild perinuclear oedema with enlarged rough endoplasmic reticulum and unusual cytoplasmic distribution of tonofilaments were found (Fig. 1). The tonofibrils occasionally formed a shell-like pattern around the nuclei (Fig. 2). Moreover, a decreased amount of keratohyaline granules was seen. The remaining granules were smaller and their density was reduced and appeared irregular (Fig. 3).

The number and distribution of melanocytes did not reveal any significant changes in AA-treated skin in comparison with the corresponding placebo site of each individual, as well as when comparing the collective samples of all individuals (Table I). Marked cytoplasmic alterations were not observed, although some swollen mitochondria with loss of the cristae and matrix were regularly present (Fig. 4). A few melanocytes also revealed an increased number and density of Golgi-associated vesicles and of cytoplasmic cisternae somehow suggesting increased synthetic activity; nevertheless, the amount of melanosomes in their cytoplasm was found to be unchanged. In some specimens, some decrease of the number of melanosome complexes in the surrounding basal and suprabasal keratinocytes was seen.

An additional light microscopic evaluation performed by two independent investigators



Fig. 2. Low malpighian layer of normal human epidermis treated with AA. Note the irregular nucleus and the shell-like arrangement of the tonofilaments (arrows). The endoplasmic reticulum is vacuolated and some mitochondria show loss of their cristae ( $\times 17\ 160$ ).

Table I. Number and distribution of melanocytes in normal human skin (collective samples evaluation) before and after topical azelaic acid (20% cream) as compared to placebo

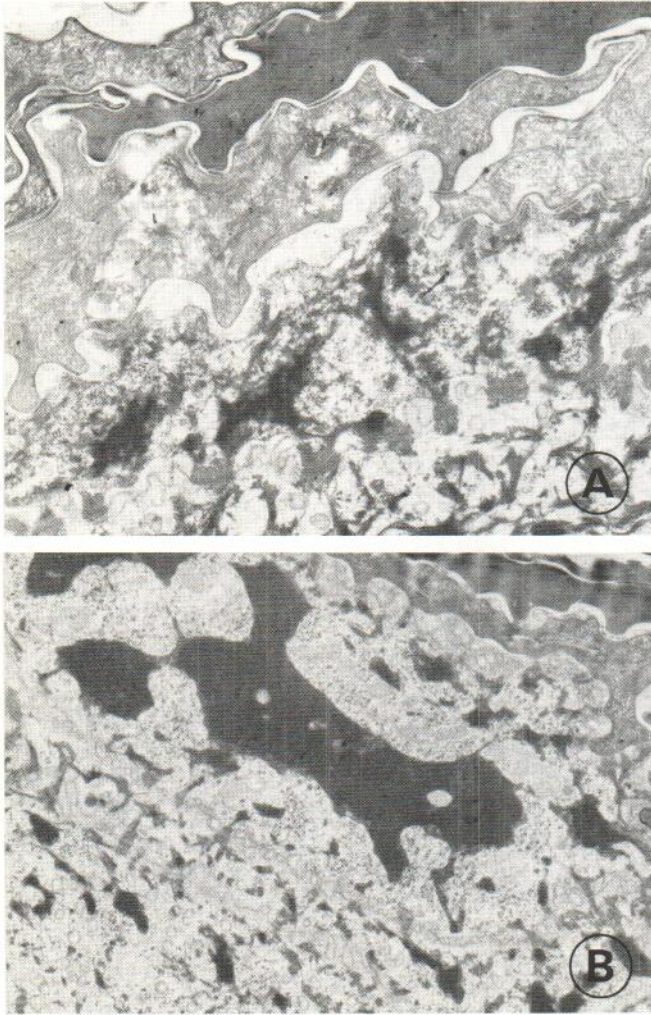
|   | Before treatment | After 3 months treatment with |                  |
|---|------------------|-------------------------------|------------------|
|   |                  | Placebo                       | Azelaic acid     |
| Number of biopsies studied                                    | 15               | 15                            | 13               |
| Number of evaluated areas of the sections                     | 47               | 47                            | 39               |
| Total number of counted melanocytes                           | 737              | 774                           | 624              |
| Total length of basement membrane (mm)                        | 19.79            | 18.87                         | 14.94            |
| Number of melanocytes per mm of basement membrane ( $\pm$ SD) | 37.35 $\pm$ 5.58 | 42.04 $\pm$ 5.98              | 42.13 $\pm$ 7.18 |

Comparison placebo/azelaic acid  $p \leq 0.44$  (non significant).

Comparison before/azelaic acid  $p \leq 0.018$ .

Comparison before/placebo  $p \leq 0.03$ .





*Fig. 3.* In the stratum corneum incomplete pattern of keratinization and irregular keratohyaline granules are seen in AA treated skin (A), compared to control skin (B). The size of the granules is smaller and their structure appears less dense and less homogeneous (A:  $\times 16\,920$ ; B:  $\times 13\,380$ ).

using silver impregnated slides from 12 treated individuals, revealed that the melanin content seemed rather decreased at the verum site in 9 individuals and was rather increased in 2; no difference was seen in one individual, as compared to placebo.

The epidermal intercellular spaces frequently contained extracellular exsudate, also showing fine osmiophilic granules, most likely corresponding to proteins. The plasma membranes of all epidermal cells remained mostly intact after AA-application.

## DISCUSSION

These results indicate that long-term topical application of AA may alter the ultrastructural characteristics of normal human keratinocytes, thus mildly affecting the keratinization process. In particular, the electron microscopic findings clearly suggest that the number and size of keratin precursors, such as tonofilaments and keratohyaline granules, will be reduced in normal human skin, if AA is applied topically twice daily over a period of 3 months. The decreased and irregular density of the remaining keratohyaline granules

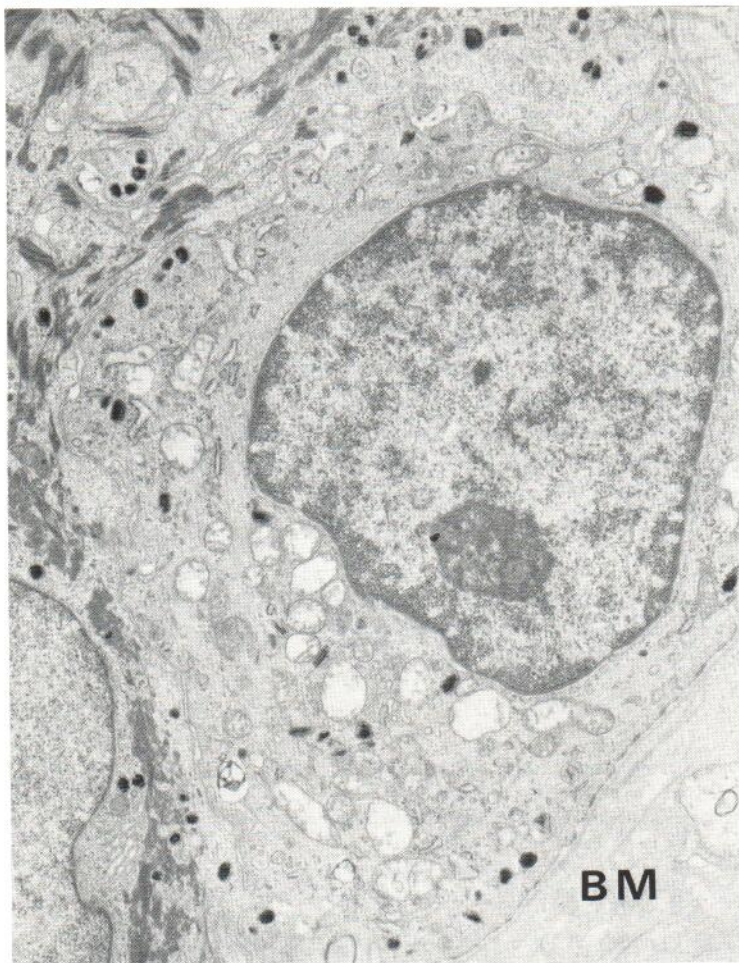


Fig. 4. Well preserved melanocyte in AA treated skin. Only some swollen mitochondria with loss of cristae are regularly seen. The number and size of melanosoma appear normal (BM=basement membrane) ( $\times 14\ 810$ ).

suggests a modulating influence of the drug during the terminal phases of keratinocytic differentiation. In regard to the abnormal distribution of tonofilaments seen in the malpighian layers one may suspect that AA possibly also alters earlier stages of the keratinization process.

In addition to the ultrastructural findings in human skin presented here, other investigations from our laboratories recently showed that AA obviously inhibits proliferation and modifies protein synthesis of mouse keratinocytes, particularly the synthesis of keratohyaline-associated macroaggregates and of non-crosslinked proteins (8, 9). Such effects may also be caused by other saturated dicarboxylic acids ( $C_6$ ,  $C_9$ ,  $C_{10}$ ) to varying degrees (10).

Besides the alterations of keratin precursors, mitochondrial swelling was a constant observation in specimens treated with AA. Corresponding findings were reported in melanocytes cultured *in vitro* under the presence of this drug (11, 12). Osmotic effects seem unlikely, since the mitochondrial envelopes remain normal, whereas the mitochondrial cristae clearly disappear, indicating some effect of AA on mitochondrial metabolism, as has been suggested by others (12, 13).

To what extent the swelling of mitochondria may influence the keratinization process remains subject for further elucidation.



The number and distribution of melanocytes did not reveal any specific change in normal skin under the application of AA, in contrast to observations on malignant melanocytes (14). The number of melanocytes in both the verum and the placebo sites had increased, however, as compared to the untreated controls. It seems that the daily mechanical rubbing over 3 consecutive months may have caused irritation and some hyperpigmentation of the treated skin that obviously remained subclinical.

Our findings also suggest that AA can have, to some extent, an influence on melanocytic cell activity, since the Golgi associated vesicles and membranes were increased and the melanosome complexes in surrounding keratinocytes seemed decreased, but these slight changes also remained at subclinical levels.

In conclusion, the electron microscopic findings collected carefully in a double blind-manner comparing AA vs. placebo suggest that AA may modify keratinocytic differentiation in normal human skin by reducing the synthesis of keratin precursors. This effect could play a major role in the beneficial clinical effect of AA in acne, by normalizing the disorder of keratinization in the acroinfundibulum. No clear-cut changes caused by AA appeared in normal human melanocytes after 3 months of local treatment.

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#### REFERENCES

1. Nazarro-Porro M, Passi S, Picardo M, Breathnach A, Clayton R, Zina G. Beneficial effect of 15% azelaic acid cream on acne vulgaris. *Br J Dermatol* 1983; 109: 45-48.
2. Picardo M, Passi S, Nazarro-Porro M, Breathnach A. Effect of medium chain length dicarboxylic acid on mitochondrial respiration. *J Invest Dermatol* 1983; 80: 350.
3. Passi S, Picardo M, Nazarro-Porro M, Breathnach AS, Confaloni AM, Serlupi-Crescenzi G. Anti-mitochondrial effect of saturated medium chain length ( $C_8-C_{13}$ ) dicarboxylic acids. *Biochem Pharmacol* 1984; 33: 103-108.
4. King K, Leeming JP, Holland KT, Cunliffe WJ. The effect of azelaic acid on cutaneous microflora in vivo and in vitro. Paper presented at the 15th annual meeting of the E.S.D.R., Amsterdam, May 12-15th, 1985.
5. Nazarro-Porro M, Passi S, Picardo M, Breathnach AS. Possible mechanism of action of azelaic acid on acne. Paper presented at the 15th annual meeting of the E.S.D.R., Amsterdam, May 12-15th, 1985.
6. Breathnach AS, Nazarro-Porro M, Passi S. Azelaic acid. *Br J Dermatol* 1984; 111: 115-120.
7. Gassmüller H, Graupe K, Orfanos CE. Azelaic acid and sebum excretion rate. *Br J Dermatol* 1985; 113: 800.
8. Müller R, Stadler R, Detmar M, Orfanos CE. Azelaic acid inhibits proliferation and modifies differentiation of neonatal mouse keratinocytes in vitro (submitted for publication 1986).
9. Mayer-da Silva A, Müller R, Gollnick H, Orfanos CE. Azelaic acid inhibits proliferation and modulates differentiation of keratinocytes in vivo and in vitro. *J Cutan Pathol* (in press).
10. Detmar M, Müller R, Stadler R, Orfanos CE. Dicarboxylic acids modulate protein synthesis and inhibit proliferation of keratinocytes in vitro. *J Invest Dermatol* 1986; 87: 136A.
11. Robins E, Breathnach A, Bennett D, Ward B, Bhasin J, Etheridge L, Nazarro-Porro M, Passi S, Picardo M. Ultrastructural observation on the effect of azelaic acid on normal human melanocytes and a human melanoma cell line in tissue culture. *Br J Dermatol* 1985; 113: 687-697.
12. Hu F, Mah K, Teramura DJ. Effects of dicarboxylic acids on normal and malignant melanocytes in culture. *Br J Dermatol* 1986; 114: 17-26.
13. Ward BJ, Breathnach AS, Robins EJ, Bhasin J, Passi S, Nazarro-Porro M. Analytical, ultrastructural, autoradiographic and biochemical studies on  $^3H$  dicarboxylic acid added to cultures of melanoma cells. *Br J Dermatol* 1984; 111: 29-36.
14. Nazarro-Porro M, Passi S, Zina G, Bernengo A, Breathnach A, A, Gallagher S, Morpurgo G. Effect of azelaic acid on human malignant melanoma. *Lancet* 1980; i: 1109-1111.