Acta Derm Venereol (Stockh) 1993; 73: 97–101

Topical All-*trans* Retinoic Acid Rapidly Corrects the Follicular Abnormalities of the Rhino Mouse

An Ultrastructural Study

PEISHU ZHENG[1], GERARD J. GENDIMENICO[2], JAMES A. MEZICK[2] and ALBERT M. KLIGMAN[1]

[1]Department of Dermatology, University of Pennsylvania, School of Medicine, Philadelphia, PA and [2]The R. W. Johnson Pharmaceutical Research Institute Raritan, New Jersey, USA

Topical all-*trans* retinoic acid (RA) has been shown to transform the horn-filled utriculi of the rhino mouse into normal follicles. We studied the early events by light and electron microscopy. Reduction in diameters of the utriculi was quantified by image analysis of whole mounts. Topical RA at 0.05% in ethanol/propylene glycol was applied daily and biopsies were taken after 1, 2, 3 and 6 days of treatment. By electron microscopy, after 3 days of RA treatment there was a great increase in the size and density of laminated membrane coating granules (MCGs) which had fused to the apical membranes of the upper granular cells. Thereafter, corneocytes within the lumina of the utriculi showed fewer desmosomes and a loss of intercellular material, accompanied by detachment from each other. Conversion to normal follicles was complete by 6 days. In whole mounts examined after 3 days of RA, there was a 75% reduction in the mean diameter of the utriculi. These results suggest that extrusion of the contents of enlarged MCGs into the intercellular corneocyte spaces facilitated separation of corneocytes, leading to rapid shedding, perhaps through the action of desmosome-lysing proteases. The conversion to normal follicles is consistent with the established role of retinoids in correcting abnormal differentiation. *Key words: Comedolysis; MCGs; Utriculi; Desmosomes.*

(Accepted October 28, 1992.)

Acta Derm Venereol (Stockh) 1993; 73: 97–101.

P. Zheng, Department of Dermatology, University of Pennsylvania 422 Curie Blvd., Phila., PA 19104, USA.

The rhino mouse ($hr^{rh}hr^{rh}$) is a mutant form of the hairless mouse (hr hr). It has a grotesque appearance because of its highly redundant skin which contains deformed, non-hair bearing follicles whose orifices are distended with horny material. These structures resemble the open comedones of acne patients and are referred to as utriculi or pseudocomedones.

The rhino mutant has become a useful model for assaying the comedolytic activity of topical drugs (1–6).

Kligman & Kligman applied tretinoin daily for 6 weeks and found that all-*trans* retinoic acid (RA) not only emptied the utriculi of horn but also restored the follicles to a near-normal appearance (2). The comedolytic effect was dose-dependent and was accompanied by a marked thickening of the epidermis and a prominent granular layer. These effects are typical of retinoid action. Other agents, such as salicylic acid, caused partial emptying of the horny masses, without evidence of follicular normalization. Lactic acid, propylene glycol and benzoyl peroxide had little or no effect on the horny contents of the utriculi.

The Kligmans assessed comedolytic activity histologically on transverse sections. At best, this yields only semi-quantitative results. Bonne et al. likewise evaluated comedolytic activity histologically, expressing comedolytic potency as the ratio of the orifice of the utriculus to its widest diameter (3). This procedure is more quantitative but laborious. Mezick and co-workers (4) overcame these limitations by preparing whole mounts of epidermis which were then rendered transparent (4). It was then possible to scan the specimen by image analysis to determine the mean diameter of a large number of utriculi, which normally vary greatly in size. They applied RA, isotretinoin (13-*cis*-RA), motretinide and etretinate topically for 2 weeks and also orally for 3 weeks. They found a substantial reduction in utriculi diameters after topical RA; the other retinoids were considerably less potent. At an oral daily dose of 5 mg/kg, each retinoid significantly reduced the mean diameter; again RA was the most effective. Using the whole mount method, Ashton et al. assayed the effect of 2 weeks of daily application of RA, 13-*cis*-RA, etretinate and an arotinoid ethyl ester (5). These produced dose-dependent reductions in the size of the utriculi, accompanied by thickening of the interfollicular epidermis and the follicular epithelium. Moreover, the skin reverted to the architecture of hairless mice as reported earlier with RA (2). Chatelus et al. showed that the rhino mouse was a reliable model for ranking the therapeutic efficacy of retinoids (6). Recently Bernard et al. used electron microscopy to describe the ultrastructural changes induced by 3 weeks of application of 0.025% RA and a new synthetic retinoid (CD271) (7). Both effected a marked reduction of the diameter of utriculi with normalization of the follicles. After 2 days of RA treatment, mast cells became degranulated and lymphocytes were found in contact with shrunken Langerhans' cells (8). After 1 week the number of MCGs had increased greatly, associated with disorganization and shedding of the horny layer. They postulated that the comedolytic effect resulted from increased proliferation and altered differentiation of follicular keratinocytes.

The above studies mainly described the changes after 2 to 3 weeks of RA treatment. We thought it worthwhile to pay more attention to early events in order to understand better the mechanism of comedolysis. We were able to demonstrate that the comedolytic effect of RA begins within a few days and that these early morphologic events are guite different from those observed after longer periods.

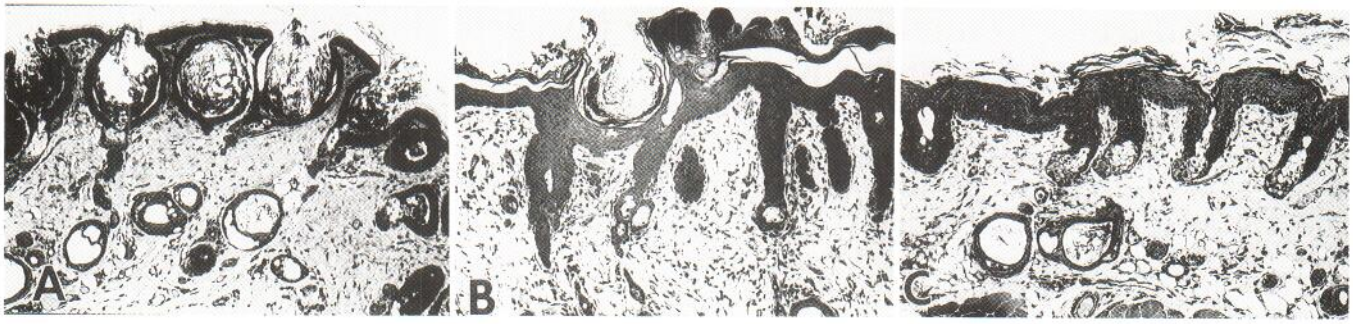

Fig. 1. (A) Vehicle-treated skin showing a dense row of horn-filled utriculi resembling open comedones. Much of the horny impaction has been lost during processing, creating an artifactual appearance of half-empty utriculi (JB4 × 60). (B) After 3 days of RA, the horny impactions have mostly been extruded. The utriculi are being transformed into normal follicular structures. Note increased density of cells in the dermis, chiefly enlarged fibroblasts (JB4 × 60). (C) After 6 days of RA normalization is complete with no sign of retained horn in the follicular orifices. The epidermis is greatly thickened as is the follicular epithelium. The sebaceous glands are larger. Dermal cellularity is further increased with many hypertrophic fibroblasts (JB4 × 60).

MATERIAL AND METHODS

Treatment

Male rhino mice, 8–10 weeks old, were studied. A volume of 0.1 ml of 0.05% RA in ethanol/propylene glycol (70:30) was applied once daily to the dorsal trunk of groups of 4 mice each for 1, 2, 3 and 6 consecutive days. Separate control groups were treated similarly with vehicle.

Light microscopy

The tissues were fixed in formalin, dehydrated through ethanol and infiltrated overnight in a monomer solution of JB4 water-soluble methacrylate plastic (Polysciences, Fort Washington, Pa). After embedding in JB4, two micron sections were cut with a glass knife on a Reichert-Jung 2050 Supercut microtome (Cambridge Instruments, Buffalo, NY). The specimens were then stained with toluidine blue and basic fuchsin.

Transmission electron microscopy

Tissues were fixed in paraformaldehyde-glutaraldehyde solution for 2 h at room temperature, post-fixed in osmium tetroxide, dehydrated in a series of cold ethanol and embedded in Tab Epon 812. Ultrathin sections were cut with a Porter-Blum II microtome and viewed with a Hitachi 700 electron microscope under 75 kv, as previously described (9).

Utriculi measurements

Whole mounts were prepared according to the method of Mezick et al. (4). The diameters of 50 utriculi were measured per specimen. These were randomly selected and quantified using image analysis (JAVA, Jandel Scientific).

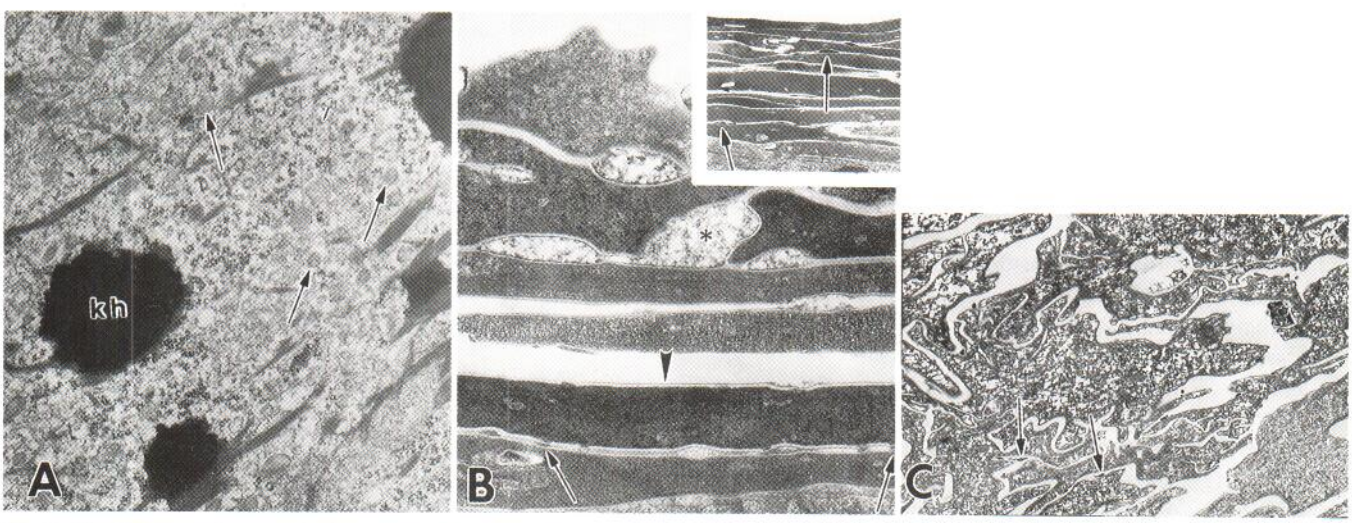

Fig. 2. (A) Electron micrograph of granular cell. Vehicle control. MCGs (*arrow*) are structureless. Keratohyaline granules (*kh*) are large with irregular shape (× 20,000). (B) Electron micrograph of corneocytes within the utriculus wall. Vehicle control. The thick cell envelope (*arrowhead*) can be identified. Desmosomes are plentiful in contrast to their relative absence further toward the lumen. Membrane-like (*arrow*) and granular (*) intercellular material can be found in some areas; apparently empty spaces between corneocytes are an artifact. Bilaminar membranes are not visualized with this method of fixation (× 20,000). *Inset.* Horny cells are densely packed. Desmosomes (*arrow*) are prominent (× 5,000). (C) Electron micrograph of corneocytes within the central portion of the utriculus. Vehicle control. The cell borders are strinkingly convoluted, forming interlocking patterns which resemble a jig-saw puzzle. Remnants of desmosomes (*arrow*) can still be seen (× 5,000).

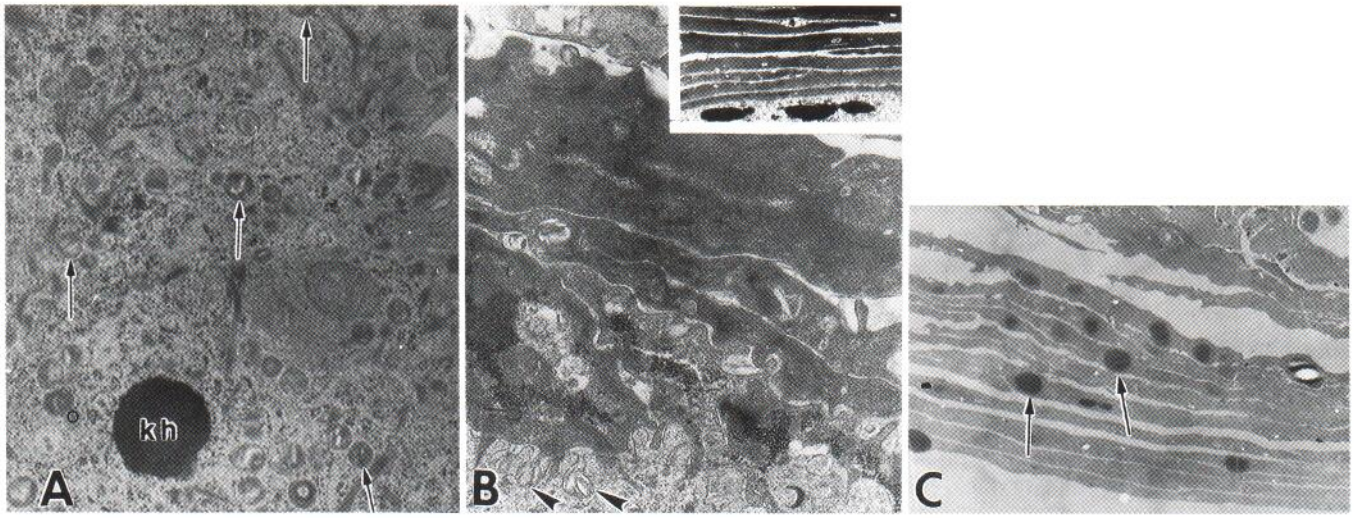

Fig. 3. (A) Electron micrograph of a granular cell of the utricular epithelium after 3 days of RA. MCGs (*arrows*) are numerous and large, showing a banded internal structure. Keratohyaline granule (*kh*) is small and round (× 20,000). (B) Three-day RA-treated skin. Many discharged MCGs have fused with the cell membranes of the granular cells at the bottom of the micrograph (*arrowhead*). The newly formed corneocytes are flat, with very narrow intercellular spaces, apparently reflecting rapid loss of the lipoidal intercellular cement (× 20,000). (*Inset*). Corneocytes are flat and are devoid of desmosomes (× 5,000). (C) Three-day RA-treated skin. Electron-dense lipid droplets (*arrow*) indicate greatly increased proliferative activity of the follicular epithelium. The narrow, flattened corneocyte layers in the bottom have been newly formed. Older corneocytes toward the center of the utriculus are swollen, distorted and separating (× 5,000).

Transepidermal water loss (TEWL)

TEWL was measured with a SERVO-MED evaporimeter (Model EP1) under ambient laboratory conditions.

RESULTS

Light microscopy

Vehicle. No changes were seen with the vehicle at any of the time periods. The follicular epithelium consisted of 3–4 cell layers. The horny material in the utriculi was comprised of multiple layers of corneocytes. Small sebaceous glands were attached to the base of the utriculi (Fig. 1A).

All-trans *retinoic acid (RA)*. Application of RA for 1 day had no effect. However, by 2 days the contents of the utriculi had begun to diminish and the diameters were consequently smaller. The follicular epithelium thickened to 4–6 layers. The horny impactions appeared looser and were partially expelled. After 3 days, there was a tendency toward normalization of many follicles. The follicular epithelium and the epidermis were considerably thicker, reaching up to 10 cell layers. Horny material was mostly expelled. Because of the greatly narrowed dimensions of the utriculi, there were long stretches of epidermis which were free of follicular structures. Dermal cellularity increased greatly. The new complement of cells consisted mainly of fibroblasts and macrophages (Fig. 1B). After 6 days, all utriculi had been converted to normal follicular structures. The sebaceous glands were modestly enlarged with more sebocytes per lobule. The epidermis was about 10 cells thick with widened intercellular spaces and a loose thin, horny layer (Fig. 1C).

Electron microscopy

Vehicle. The skin was not affected even after 6 days. The follicular epithelium consisted of 3–4 layers, including the granular layer which was 1 or 2 cells thick with large, irregularly shaped keratohyaline granules. Membrane coating granules (MCGs) were mostly scanty. Their laminated internal structure was not well-defined (Fig. 2A). The horny layer was coherent, 5 or 6 cell layers thick with well preserved desmosomes. Lamellar and granular material filled the intercellular spaces (Fig. 2B). In the central lumina of the utriculi, the horny cells were arranged in an interlocking pattern and often had a jigsaw-puzzle appearance. Intercellular granular material and remnants of desmosomes were discernible (Fig. 2C).

All-trans *retinoic acid (RA)*. After 3 days the epithelium of the utriculi thickened to 4–6 cell layers. The intercellular spaces were widened and filled with fine granular material. Increased mitoses were found in the basal layer. The granular layer became 3 cells thick but had fewer and smaller keratohyalin granules than in controls. The number and size of MCGs had greatly increased (Fig. 3A). Most of these had a well-defined laminated structure. Many were seen to have fused with the upper cell membrane of the outermost granular cells, discharging their contents into the intercellular space (Fig. 3B). The newly-formed horny cells adjacent to the utricular epithelium had fewer desmosomes. The intercellular spaces between corneocytes seemed emptier. The corneocytes had thinner cell envelopes (marginal bands), were flattened and contained numerous lipid droplets (Fig. 3C). Tonofilaments were sparse.

After 6 days the utriculi had emptied completely. The epidermis had the usual RA appearance, i.e. hyperplasia and widened intercellular spaces filled with fine granular material. MCGs were numerous, large, and well laminated. These had fused with the cell membranes and were discharging their contents into the intercellular spaces at the granular-horny cell junction.

Utriculi diameter in whole mounts (RA)

A marked decrease (76.5%) occurred after 3 days without further changes by 6 days (75.5%).

Transepidermal water loss (TEWL) (RA)

After 2 days, TEWL increased about 4-fold. There was a greater than 7-fold increase after 3 days and a 9-fold increase by 6.

DISCUSSION

The salient finding in this study is the magnitude of the changes seen as early as after 2 days of RA treatment. Indeed, in whole mounts the size of the utriculi had already shrunken and after 3 days of treatment, the utriculi were maximally reduced in size.

The major histologic changes after 3 days of RA treatment were hyperplasia of the utricular wall, loosening of horny material and a normalization of follicular structures. The end result is the transformation of the architecture of rhino mouse skin to that of the hairless mouse.

The morphologic changes observed by transmission electron microscopy enable one to speculate about the mechanisms by which RA leads to rapid loss of cohesiveness between corneocytes. The key finding was a marked increase in the number of enlarged MCGs followed by massive discharge of their contents into the intercellular spaces. It is universally accepted that lipids account for the barrier function of the stratum corneum (10, 11). After extrusion, the contents of the MCGs are rearranged into broad sheets of bilaminar membranes (12). This structural arrangement is indispensable to barrier function and is absent in disorders where the barrier is dysfunctional.

Biochemical and histochemical analysis of purified MCGs shows these to contain a heterogeneous mixture of complex molecules. The lipid fraction consists mainly of glycolipids, phospholipids and cholesterol (13).

The role that lipids play in the cohesion of corneocytes is more controversial. Both increased and decreased cell cohesion have been reported (6, 14–16).

On the other hand, it has been amply demonstrated that MCGs contain an astonishing variety of catabolic enzymes including proteases, lipases, glycosidases, and acid phosphatases (17–19). The presence of these enzymes is the reason why MCGs are sometimes viewed as lysosomes (17, 20).

We found a great increase in MCGs in RA-treated skin, but paradoxically the intercellular material decreased. A possible explanation is that the lipids were hydrolytically degraded by lipolytic enzymes discharged by exocytosis of MCGs. Less controversial is the role of desmosomes in the bonding of corneocytes. These attachment plaques are prominent in newly-formed horny cells; they practically disappear near the surface where desquamation begins. This appears to be an enzymatically-mediated event in which proteases, derived from MCGs, attack desmosomal proteins (21, 22). Indeed, antiproteases have been shown to prevent the dissolution of desmosomes in vitro (22).

We found a rapid depletion of desmosomes after RA treatment. Loosening of the cohesion between corneocytes would inevitably lead to extrusion of the pseudocomedones. The same effect of retinoids on desmosomes has been reported by other authors (23–25). We saw massive disgorgement of MCGs into the intercellular domains of the lower horny layers. A great influx of the contents of MCGs would quickly lead to structural disassembly of the corneocytes comprising the pseudocomedones.

Changes in the sturdiness of the corneocytes could also affect cell cohesion. After RA treatment, the horny cells became flattened with thinner envelopes. Vacuoles with the morphology of lipids were seen in these corneocytes, a feature usually associated with increased proliferation.

It is noteworthy that premature shedding of corneocytes with thinning of the horny layer is characteristic of RA-treated human skin (23–25).

We found more laminated MCGs after RA treatment, as did Maeda (26). Enhanced desquamation has also been reported by others, always accompanied by drastic changes in the morphology of MCGs (29).

In view of the complex actions of retinoids on epithelial tissue, no simple explanation will suffice. Rapid exfoliation of pseudocomedones does not explain the conversion of utriculi to normal follicles.

The rhino mouse is a convenient model for assaying comedolytic effects. The similarity of the effects of RA on rhino mouse skin to that which occurs in human skin makes the results relevant. Whether one measures utriculi diameters in whole mounts or assesses utriculus size in histologic sections, it would appear that the 3-week treatment period customarily used to assess comedolytic activity is unnecessarily long.

Our findinqs are generally congruent with those of other workers in this field. Further morphological studies will probably not be fruitful. Instead biochemical studies will probably explain how retinoids accelerate the process of desquamation.

ACKNOWLEDGEMENTS

We are deeply indebted to Lorraine H. Kligman for her valuable discussion and suggestions in this work. We thank John P. Mallon and Sijie Zheng for their excellent technical assistance.

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