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Ultrastructure of Murine Epidermis Treated with the Vitamin D_3 Analogue KH-1060

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A new highly potent analogue (KH-1060) of vitamin D_3 has been recently shown to stimulate the growth and differentiation of keratinocytes. This study was intended to determine the effects of this new analogue on epidermis at the ultrastructural level. KH-1060 was applied topically on the backs of hairless mice for 4 weeks; the skin was then studied by routine electron microscopy. The effects were compared with those of betamethasone-17-valerate and with concomitant treatment of KH-1060 following betamethasone. KH-1060 stimulated normal function of keratinocytes and formed a thick epidermis. The ultrastructure of the thick epidermis represents an enhanced normal process of keratinization and proliferation. Moreover, KH-1060 diminished the atrophogenic effects of betamethasone. ***Key words: keratinization; proliferation; hairless mice.***

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1α,25-dihydroxyvitamin D_3 (calcitriol) enhances orthokeratotic keratinization of murine and human keratinocytes in vitro as well as in vivo, although proliferation of keratinocytes has been reported to be inhibited by calcitriol in vitro (1,2). KH-1060 is a new analogue of calcitriol with a higher potency to regulate the growth and differentiation of lymphoma cells than calcitriol (3). The analogue arrests the life cycle of keratinocytes in the Sphase and stimulates keratinization (4). Using ultrasonography and light-microscopical morphometry, we have previously described how KH-1060 thickened the epidermis of hairless mice, which is beforehand atrophied by betamethasone, both remedies being applied topically (5).

This study is intended to describe the ultrastructural patterns of the keratinization process of hairless mice skin under the influence of KH-1060 in comparison with the effects of corticosteroids.

MATERIAL AND METHODS

Animal experiments

Briefly, 56 young female hairless mice, about 20 gm in weight, were divided into four groups and used for the experiments. The first group was treated with the buffered solution of KH-1060 (20-epi-22-oxa-24a-homo-26,27-dimethylcarcitriol (Leo Pharmaceutical Products, Denmark) in isopropanol (0.2 mcg/ml), the second with betamethasone-17-valerate (Betnovat®, Glaxo, UK) solution in isopropanol (0.2 mg/ml), and the third with the betamethasone solution followed by the KH-1060 solution. The fourth group was with the vehicle, isopropanol, only. Fifty microliters of the solutions for each mouse were dropped on the backs of the mice in every group and dried in air, once a day for 4 weeks. For the third group, the KH-1060 solution was dropped in the same area after drying the betamethasone solution. The fourth group was for control, using isopropanol solution, dropped once for groups 1, 2 and 3, and repeated once more for group 4. Skin specimens were obtained from the painted areas at the end of the experiments by a 4-mm punch.

Morphometry in light microscopy

Formol-fixed paraffin sections were stained by hematoxylin eosin. By light microscopy the thickness of the epidermis was measured in the areas showing flat papillae between adjacent hair follicles. Thickness was measured from the level immediately under the horny plates to the basement membrane. The numbers of the cell layers in strata granulosa and spinosa were counted.

Preparation for electron microscopy

Skin specimens were fixed in 4% glutaraldehyde solution in cacodylate buffer, pH 7.4, with 7.5% sucrose. After osmification, the specimens were dehydrated and embedded in epoxy resin. Ultrathin sections were stained by uranyl acetate and lead citrate. An electron microscope, JEOL 100 CX, operated at 80 kv, was used for the study.

Energy dispersive X-ray microanalysis was performed on the routinely prepared ultrathin sections for detection of calcium in the large matrix granules (kindly performed by Dr Morten Nielsen, Pathological Anatomy Department, University of Copenhagen).

RESULTS

A. Morphometry in light microscopy

Epidermis was thickened in the KH-1060-treated group and in the group of KH-1060 treatment following betamethasone treatment. Thickening was caused by the polygonal and oval shapes of the keratinocytes as well as the increased numbers of the keratinocyte layers. Sole treatment by betamethasone resulted in thin epidermis, which consisted of a few cell layers of flat keratinocytes (Table I).

B. Electron microscopical findings

Compared with the earlier publications on the ultrastructure in normal epidermis of adult hairless mice (6, 8, 11), epidermis of young hairless mice in the vehicle group showed some different findings, as described below. A survey of the findings in the four experimental groups is found in Table II.

1. Vehicle group. Desmosomes were few, lacking median lines. The spinous cells were jointed by numerous finger-like protrusions of cytoplasm. The cells contained sparse tonofilament bundles. The keratinocytes in the basal cell layer contained no matrix granules in the mitochondria and showed numerous ribosomes forming rosettes.

2. KH-1060 group. The findings different from the vehicle group were the following. Orthokeratotic corneocytes were seen in multiple layers. Parakeratotic corneocytes were rarely found (Fig. 1). Granular cells contained numerous keratohyalin granules in the forms of small droplets to large irregular shapes.

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Table I. *Morphometric evaluation (mean and SD)*

Thirty representative areas of the histological specimens were evaluated in each group, with each animal in the group represented.

Epidermal thickness (mm)

Vehicle	Betamethasone	KH-1060	Concomitant
0.023±0.004	0.014±0.0016	0.062±0.013	0.054±0.012

Cell numbers in the epidermal layers

	Vehicle	Betamethasone	KH-1060	Concomitant
Granular cell layers	1.56±2.0*	1.67±2.4*	3.26±0.63**	2.84±0.64**
Spinous cell layers	2.43±2.42	1.72±3.0	3.91±0.45	3.06±0.73

* Flat granular cells.
** Oval and polygonal granular cells.

Keratinosomes were numerous and small showing internal lamellae. They showed a tendency to gather in the space under the corneocytes (Fig. 1). The desmosomes in the upper spinous cell layers revealed faint broken median lines (Fig. 1, inset). The keratinocytes of the spinous cell layers contained thick tonofilament bundles and vacuolated mitochondria with large dense matrix granules (Fig. 2). Ribosomes were numerous, forming rosettes. Nuclei showed large chromatin conglomerates with nucleoli (Fig. 2). Basal cells contained well developed tonofilament bundles, and the mitochondria were vacuolated. Mitosis was found (Fig. 3). In the dermoepidermal junction basal lamina was seen diffusely thickened with interruptions in places (Fig. 3). No calcium apatite crystals were detected in the large matrix granules by routine electron microscopy; neither was calcium detected in the large matrix granules of mitochondria by energy dispersive X-ray microanalysis. (No figures are presented because of negative results.)

3. Betamethasone group. Corneocytes were parakeratotic. The granular and spinous cells were in 1–2 layers. The cells were flat, containing a few small keratohyalin droplets. Keratinosomes were small and homogeneous, gathering under the corneocytes. Tonofilament bundles were thin and short. Desmosomes were few, lacking median lines. Basal cells contained polysomes and were poor in tonofilaments. Most of the hemidesmosomes were seen as homogeneous spots with radially arranged anchoring filaments. Basal lamina was irregularly thickened with traces of anchoring fibrils.

4. KH-1060- and betamethasone-treated group. Corneocytes were parakeratotic (Fig. 4). The keratohyalin granules varied in size and shape. Keratinosomes were numerous, with normal internal structures in the granular cells, whilst homogeneous keratinosomes were gathered under the corneocytes, looking like foamy masses (Fig. 4). The spinous cells showed thick tonofilament bundles. Desmosomes lacked median lines. Mitochondria were vacuolated without matrix granules (Fig. 5). Basal cells showed numerous ribosomes and a few thin tonofilament bundles (Fig. 5). Basal lamina was irregularly thickened with interruptions and traces of anchoring fibrils. Some of the hemidesmosomes were structureless with anchoring filaments in a radial array (Fig. 6).

Table II. *Comparison of epidermal ultrastructures between the experimental groups*

Components/Groups	Vehicle	KH-1060	Betamethasone	Betamethasone + KH-1060
Corneocytes	Sporadic parakeratotic cells	Rare parakeratotic cells	Parakeratotic cells	Parakeratotic cells
Keratohyalin	Large and small droplets	Numerous small droplets. Large irregular shapes	Small droplets and irregular shapes	Varied in sizes and shapes
Keratinosomes	Normal shape	Numerous in normal shape, under corneocytes	Small homogeneous, gathered under corneocytes	Homogeneous, under corneocytes. Normal in cytoplasm
Tonofil. bundl.	Sparse and thin	Many and thick	Thin and short	Thin in basal cells. Thick in spinous cells
Desmosomes	Few, lacking median line	Broken median line	Few, lacking median line	Few, lacking median line
Mitochondria	No vacuoles or matrix granules	Vacuoles with large matrix granules in spinous cells	No vacuoles or matrix granules	Vacuoles without matrix granules
Ribosomes	Rosettes in basal cells	Rosettes in spinous cells	Few, rosettes in spinous cells	Few, rosettes in spinous cells
Nuclei	Ordinary shapes of chromatin	Large conglomerates of chromatin. Mitosis	Ordinary shape of chromatin	Ordinary shape of chromatin
Junction structures	Unchanged	Basal lamina, diffusely thickened with interruptions	Irregular basal lamina. Structureless hemidesmosomes. Radial array of anchoring filaments. Traces of anchoring fibrils	Irregular basal lamina with interruptions. Structureless hemidesmosomes. Radial array of anchoring filaments. Trace of anchoring fibrils

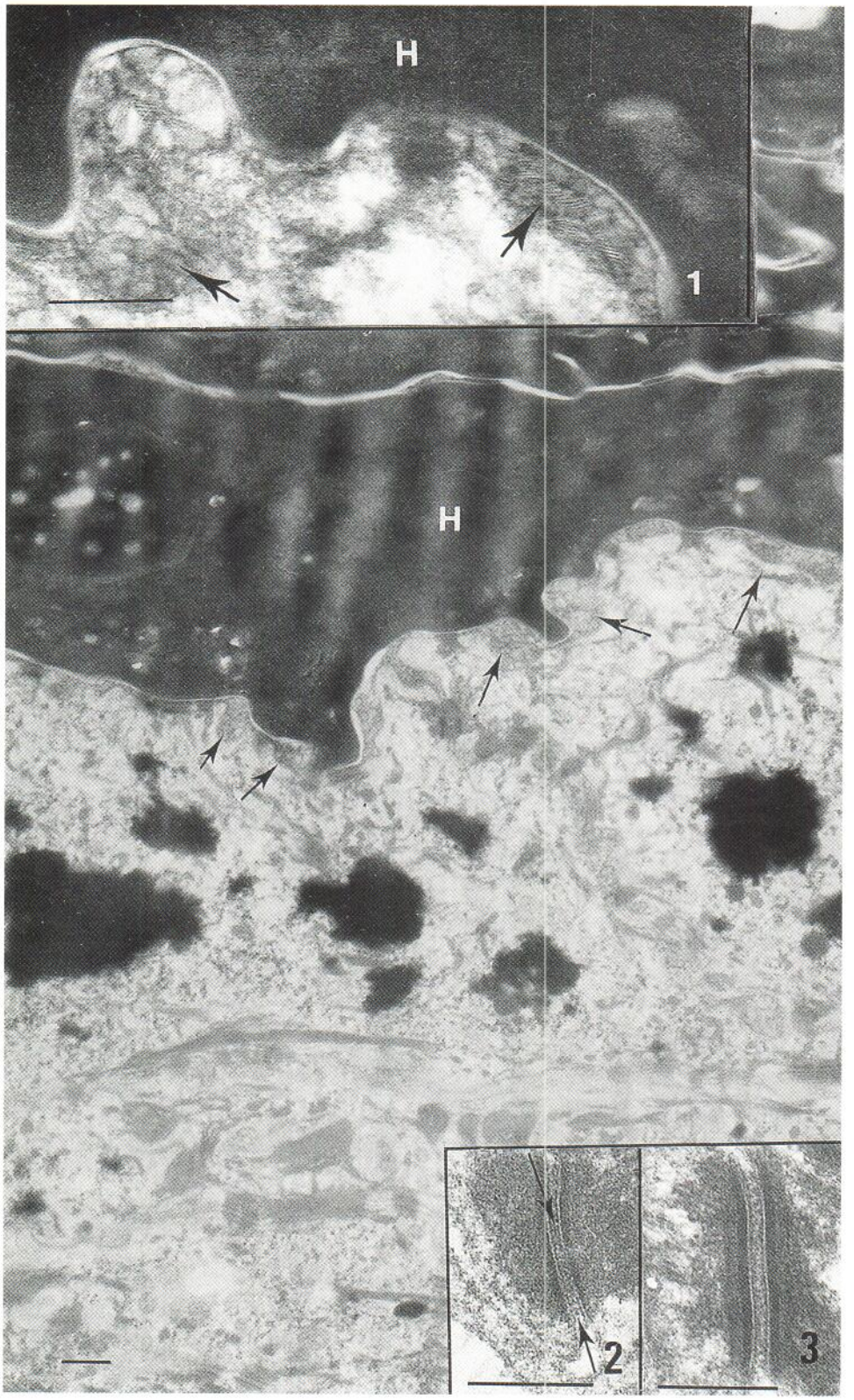

Fig. 1. The upper part of the epidermis after KH-1060 treatment. Keratohyalin granules are irregular in shapes and various in sizes. *Inset 1* shows keratinosomes with internal lamellar structures. The keratinosomes are gathered under the cell surface to the corneocytes (*arrows*). Corneocytes (*H*). *Inset 2* shows a desmosome with a faint broken median line (*two arrows*), compared with *Inset 3* from the vehicle group. ×15,000. Insets ×60,000. Scales indicate 1 and 0.1 mcm.

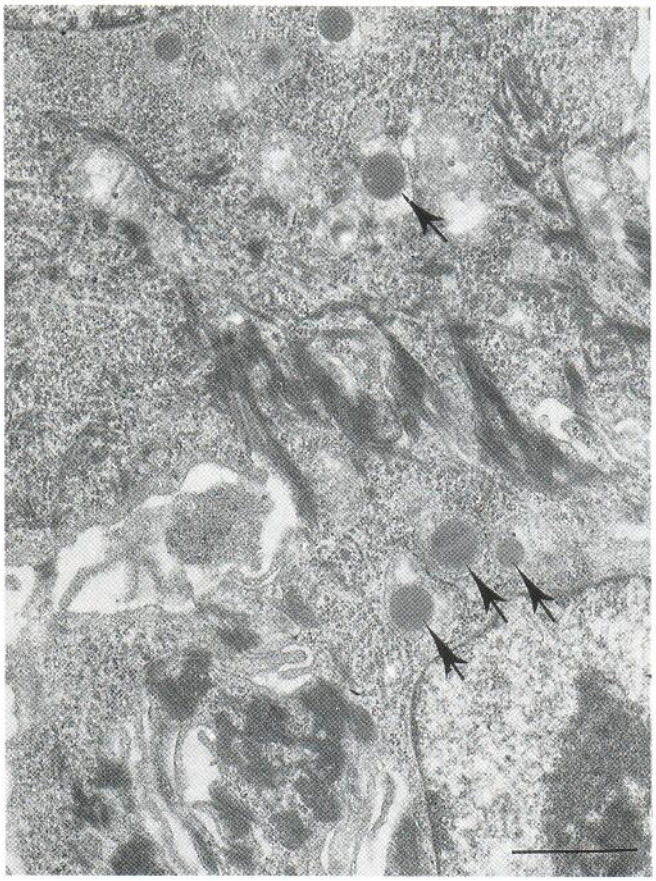

Fig. 2. The prickle cells after KH-1060 treatment. Note the large dense matrix granules of mitochondria (*arrows*). Calcium was not detected in the dense granules by energy dispersive X-ray microanalysis. ×15,000. Scale indicates 1 mcm.

DISCUSSION

Topical application of betamethasone and KH-1060 in propanol solution was effective enough to induce changes in epidermis as well as dermis, as shown in the previous report (5). For the concomitant treatment, KH-1060 solution was painted just after drying the betamethasone solution. It is therefore understood that betamethasone and KH-1060 compete as to their effects on keratinocytes. The experiments were not designed for the recovery effects of KH-1060 on epidermis beforehand atrophied by betamethasone.

When the ultrastructures of keratinization in the KH-1060-treated group were compared to those in the vehicle group, the following aspects of the KH-1060-treated group were clearly different: epidermal thickening, scarce parakeratotic corneocytes, thick bundles of tonofilament, desmosomes with faint granular median lines and large polygonal keratohyalin granules. Desmosomes lacking median lines suggest incomplete formation of desmosomes in young hairless mice. In human keratinocytes in vitro, the median line of desmosomes appeared as a broken line and developed in a continuous line (7). The broken median lines of the desmosomes in the KH-1060-treated group show that desmosome formation was stimulated by KH-1060. On the basis of these observations, KH-1060 treatment seems to stimulate orthokeratotic keratinization, as seen in adult hairless mice (6, 8–11).

Large matrix granules of mitochondria, mitosis, numerous ribosomes and interrupted basal lamina found in the KH-1060-treated group were also different from the vehicle group. Adult mice showed no special occurrence of mitosis. The mitotic rate might be increased by the KH-1060 treatment. Matrix granules of mitochondria existed in the basal cells of adult hairless mice (8); however, young ones have no matrix granules. The large matrix granules of mitochondria in the spinous cells were a remarkable finding after the sole KH-1060 treatment, though the concomitant treatment produced vacuoles without matrix granules in the mitochondria. Matrix granules take part of intramitochondrial metabolism of calcium, phosphorus and lipid (12). Calcium precipitation was supposed in the large matrix granules, hence KH-1060 penetrated through skin and elevated calcium level in serum (3) and calcium metabolism of the ker-

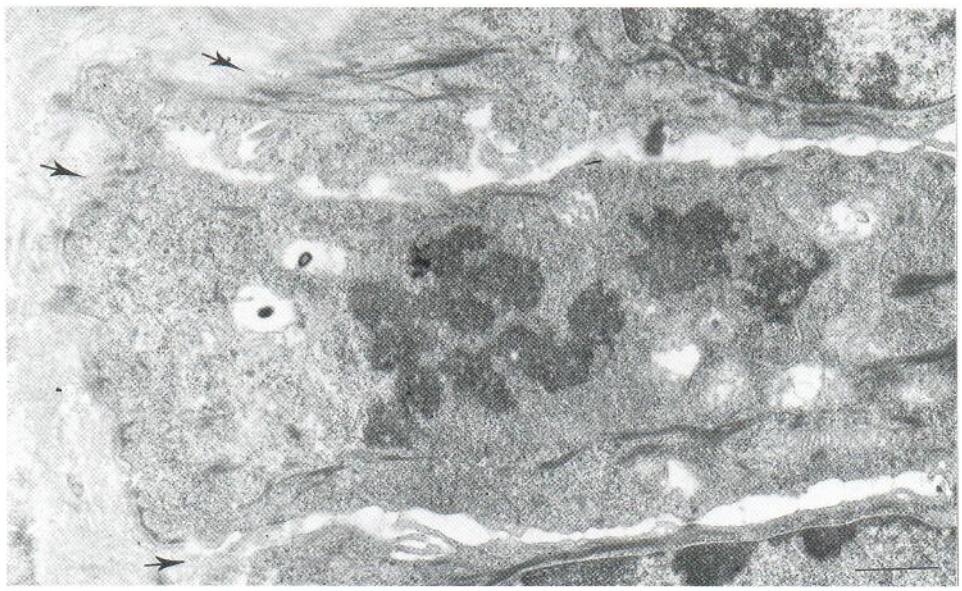

Fig. 3. The basal cells after KH-1060 treatment. The cells contain distinct tonofilament bundles. Hemidesmosomes are seen to be normal. A basal cell showed mitosis. Basal lamina is monolayer but the lamina does not border continuously. *Arrows* indicate the areas where the basal lamina is interrupted. ×15,000. Scale indicates 1 mcm.

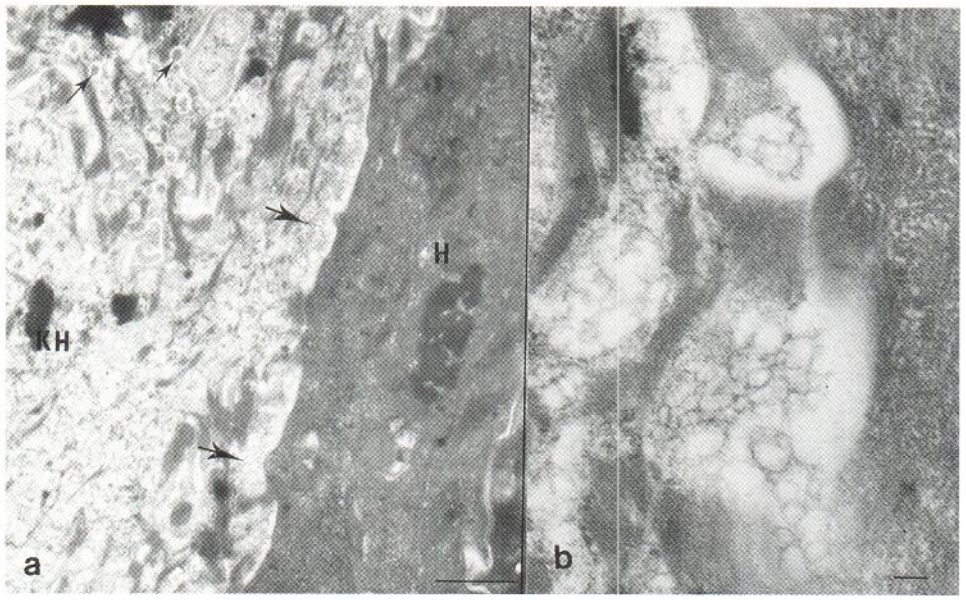

Fig. 4 a and b. The upper part of the epidermis after the treatment with betamethasone and KH-1060. The lowest corneocytes show remnants of tonofilaments and nucleus (*H*). Keratinosomes are numerous in the cytoplasm (*small arrows*) and seen to be foamy under the horny plates (*large arrows* and *4b*). Keratohyalin granules are irregular (*KH*). *a*, ×15,000. *b*, ×60,000. Scales indicate 1 mcm and 0.1 mcm, respectively.

atinocytes might be influenced by KH-1060. Although no calcium crystals were detected, electron microscopic X-ray microanalysis was further carried out for detecting minimum precipitates of calcium. However, no calcium was detected in the large matrix granules. The problem why matrix granules become larger is left unsolved. Basal lamina was a product of basal cells and always appeared as a continuous band in normal skin (13). The interrupted bands of basal lamina suggested an increased activity of the basal cells. Probably KH-1060 stimulated the cell activity of basal cells in the young mice. The present results did not seem to show any ultrastructural changes which could imply malignant transformation. The atrophic effects of betamethasone seem to be prevented by the concomitant treatment by KH-1060. The ultrastructure of the epidermis in the concomitantly treated group indicated that the keratinization and the proliferation of the keratinocytes were changing the patterns seen in adult hairless mice. These changes in the epidermis seem to be specific effects of KH-1060, since the ultrastructures did not indicate unspecific destructive changes. No unspecific inflammation was found in the dermis, and the previous paper by the co-authors has demonstrated that the chemically related analogue, 1β,25-$(OH)_2$ vitamin D_3, has produced no epidermal thickening in light microscopy (14). Light microscopically, they have also found thickening of the skin atrophied beforehand by betamethasone (14).

Compared with the earlier study on retinoid influence on the keratinocytes of adult hairless mice (15), retinoid has influenced the keratinocytes in a contrary mode, showing cytolysis in the granular cells and loss of desmosomes. Tonofilament bundles and keratohyalin granules were small in edematous cytoplasm. The normal process of keratinization may be inhibited after retinoid treatment. Probably retinoid is toxic for keratinocytes, whereas KH-1060 stimulates the metabolism of keratinocytes.

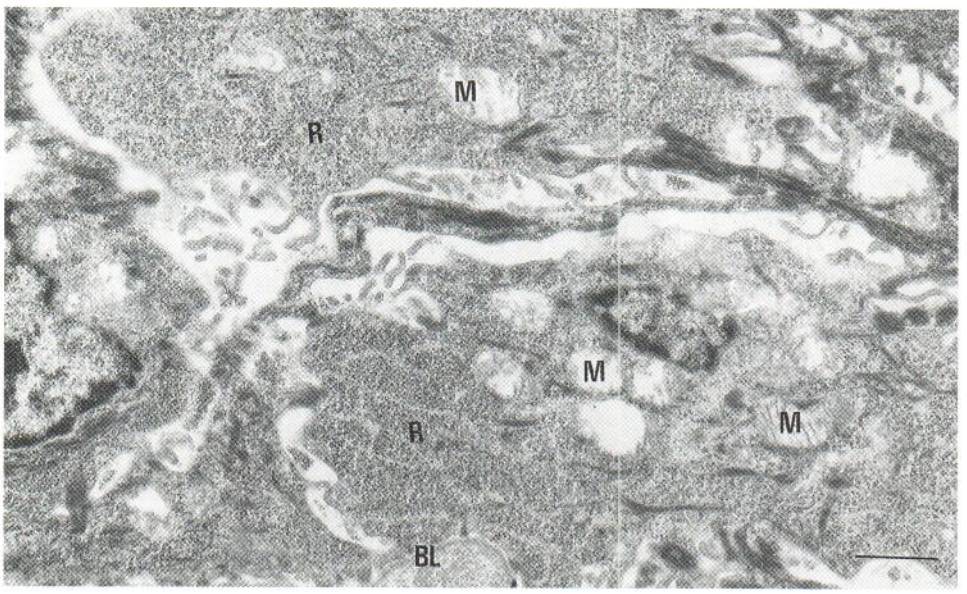

Fig. 5. The basal cells after the treatments with betamethasone and KH-1060. The basal cells show numerous ribosomes (*R*), while tonofilaments are sparse. Mitochondria (*M*) in the basal and spinous cells are vacuolated without matrix granules. Basal lamina (*BL*). ×15,000. Scale indicates 1 mcm.

Fig. 6. The dermoepidermal junction after the treatments with betamethasone and KH-1060. Note the basal lamina, of irregular thickness (*BL*) and with small interruptions (*arrows*). Some hemidesmosomes are structureless with traces of anchoring fibrils. ×30,000. Scale indicates 1 mcm.

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