Acta Derm Venereol (Stockh) 1997; 77: 198–202

Ultrastructure of Murine Dermis Following Topical Application of the Vitamin D_3 Analogue KH-1060

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The aim of this study was to observe the effects of KH-1060, a new vitamin D_3 analogue, on the dermis of hairless mice by electron microscopy. KH-1060 (0.2 μg/ml in isopropanol) or KH-1060 following betamethasone 17-valerate (2 mg/ml in isopropanol) was applied topically to the backs of hairless mice for 4 weeks. KH-1060 increased the number of dermal fibroblasts, the cytoplasm of which was dominated by secretory components. Mast cells contained normal mature granules and degranulated after disintegration. No extrusion of non-disintegrated granules was seen. Collagen fibrils were thickened and increased in number; however the content of type I collagen in the fibrils did not increase. Glycosaminoglycan figures appeared distinct. KH-1060 prevents betamethasone-induced changes in collagen fibrils and glycosaminoglycans, while no prevention was seen for mast cells. ***Key words: fibroblast; collagen fibril; glycosaminoglycans; betamethasone.***

(Accepted November 25, 1996.)

Acta Derm Venereol (Stockh) 1997; 77: 198–202.

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Calcitriol (1α,25-dihydroxyvitamin D_3) inhibits *in vitro* growth of human fibroblasts from dermis (1); however, collagen secretion is increased (2). Chen et al. (3) found that calcitriol promoted wound healing of rat skin. KH-1060 is a new potent analogue of calcitriol. Previous papers have described that KH-1060 caused thickening of the dermis of normal hairless mice and increased uptake of ^{35}S-sulphate and ^{3}H-proline (4, 5). KH-1060 enhanced the formation of granulation tissue (6) and prevented dermal atrophy by betamethasone (4). In this study, ultrastructural changes induced by KH-1060 in the dermis of hairless mice are described.

MATERIAL AND METHODS

The experiments with hairless mice have been described in a previous paper (7). KH-1060 (20-epi-22-oxa-24a-homo-26,27-dimethyl-calcitriol) was obtained from Leo Pharmaceutical Products, Denmark, and betamethasone-17-valerate was the commercial product (Betnovat®) from Glaxo, England.

The animal experiments were carried out on 56 female hairless mice, 20 g in weight, divided into four groups. Group 1 was treated with KH-1060 solution (0.2 μg/ml) in isopropanol; Group 2 with betamethasone solution (0.2 mg/ml) in isopropanol; Group 3 with concomitant treatment of the betamethasone and KH-1060 solution; Group 4 with plain isopropanol (the vehicle group). Fifty microliters of each solution were applied to the back of the mouse, once a day for 4 weeks. For the third group, KH-1060 solution was applied after drying the betamethasone solution.

For routine electron microscopy, skin specimens were biopsied in the treated areas of all the mice and fixed in a 4% glutaraldehyde solution in 0.04 M cacodylate-HCl buffer, pH 7.4, with 7.5% sucrose (Osmolarity 660 mmol). The specimens were osmicated, dehydrated and embedded in epoxy resin. Ten specimens without inflammation were selected for each group. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. The ultrathin sections were uncoded and were studied in a JEOL 100CX electron microscope at 80 kV.

For the morphometric studies of fibroblasts and mast cells by light microscopy, semithin sections were cut from the above described specimens and stained by toluidine blue. The number of fibroblasts and mast cells was counted in ten 0.1 mm^2-areas of the dermis.

For measurement of collagen fibril thickness, ultrathin sections were cut from the reticular dermis of the above described specimens. After staining, the uncoded ultrathin sections were studied by electron microscopy. Cross-sectioned compact bundles of collagen fibrils in the reticular dermis were photographed at a magnification of 20,000×. Electron micrographs used for measurement were enlarged three times. The number of collagen fibrils in each of the various size group was counted in ten 1 μm^2-areas for each group. The total number of collagen fibrils in the same areas was also counted to assess the density of the fibrils in the bundles.

For the study of collagen type I, the skin specimens were fixed in a 2% paraformaldehyde solution of phosphate-buffered saline (osmolarity 290 mmol) for 1 h at 4°C and embedded in Technovit 7100 (Kulzer, Germany). The specimens were selected in the same way as used for routine electron microscopy. Ultrathin sections of the reticular dermis were treated with polyclonal goat immunoglobulins to mouse collagen type I (Biogen, England) and detected by biotinylated immunoglobulins and streptavidin-gold technique. For control staining, unspecific goat immunoglobulins were used instead of goat immunoglobulins to mouse collagen type I. The uncoded ultrathin sections were examined by electron microscopy. Longitudinally cut, compact bundles of collagen fibrils were photographed at a magnification of 20,000×. The electron micrographs were enlarged 3 times. The number of gold particles in the collagen fibrils was counted in ten 1-μm^2 areas for both groups.

RESULTS

After KH-1060 treatment, the dermis contained numerous fibroblasts and mast cells (Table I, Fig. 1). A comparison of the dermal ultrastructure among the four experimental groups

Table I. *Numbers of fibroblasts and mast cells in dermis (0.01 mm^2)*

($n=10$)	Mean ± standard deviation	
	Fibroblasts	Mast cells
Vehicle-treated group	25.4 ± 4.2	6.2 ± 1.9
KH-1060-treated group	49.6 ± 4.5	5.0 ± 1.2
	$0.01 > p > 0.02$	$0.5 > p > 0.1$
Betamethasone-treated group	19.6 ± 2.6	5.4 ± 1.3
Betamethasone- and KH-1060-treated group	28.8 ± 5.8	4.6 ± 0.9
	$0.01 > p > 0.02$	$0.5 > p > 0.1$

 ISSN 0001-5555

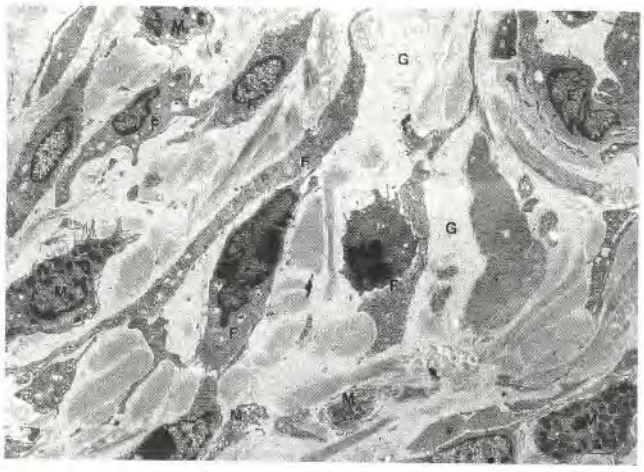

Fig. 1. Dermis after KH-1060 treatment. The dermis shows many fibroblasts (*F*) and mast cells (*M*) with or without honeycomb structure. The cytoplasm of endothelial cells (*E*), the pericytes (*P*) of vessels and fibroblasts show vacuoles in the mitochondria. Interfibrous spaces are rich in glycosaminoglycan filaments (*G*). ×6,000. *Bar*: 1 μ.

is shown in Table II. Morphometric results of collagen fibrils are shown in Tables III and IV and Fig. 4.

KH-1060 treatment increased the number of fibroblasts, the cytoplasm of which contained well-developed granular endoplasmic reticulum (Figs. 1, 2). These effects were also found in the group treated with both KH-1060 and betamethasone. Collagen fibrils were thickened and formed compact bundles after KH-1060 treatment (Figs. 3, 4). KH-1060 also thickened collagen fibrils in the group treated concomitantly with betamethasone (Fig. 4). No ultrastructural signs of degradation of collagen fibrils (9) were found in the dermis after KH-1060 treatment. All four experimental groups contained twisted

Table III. *Numbers of collagen fibrils per μm² of collagen fibril bundles*

($n=10$)	Mean ± standard deviation
Vehicle-treated group	57.9 ± 15.3
KH-1060-treated group	79.4 ± 4.0
	$p<0.05$
Betamethasone-treated group	102.5 ± 13.5
Betamethasone and KH-1060-treated group	76.9 ± 15.7
	$p<0.05$

Table IV. *Collagen type I in collagen fibrils*

The gold particles (10 nm) indicate type I collagen molecules. Number of gold particles are counted in seven 10.0-μm² areas in the closely packed bundles.

	Mean ± standard deviation
Vehicle group	97.7 ± 38.6
KH-1060-treated group	96.6 ± 22.8
	$p>0.5$
Betamethasone-treated group	60.7 ± 2.9
Betamethasone and KH-1060-treated group	63.8 ± 14.0
	$p>0.5$

Table II. *Comparison of dermal ultrastructures among the experimental groups*

Components/ groups	Vehicle	KH-1060	Betamethasone	Betamethasone and KH-1060
Fibroblasts	Sparse cell organelles	Numerous granular endoplasmic reticula & ribosomes. Few cytoplasmic filaments, lysosomes & pinocytotic vesicles (Fig. 2)	Sparse cell organelles	Same as KH-1060 treatment
Mast cell granules	Homogenous & grainy granules. Disintegration & honeycomb structures. Extrusion	Homogeneous granules. Disintegration & honeycomb structures. No extrusion	Many grainy granules. Disintegration & honeycomb structures. Extrusion	Many grainy granules. Disintegration & honeycomb structures. Extrusion
Cells in vessels & nerves	Normal mitochondria	Vacuoles in mitochondria	Vacuoles in mitochondria	Vacuoles in mitochondria
Collagen fibrils	Ordinary	Thickened. Increased in number. Compact bundles	Many thin fibrils. Loosened bundles	As thick as in the vehicle. Slight increase in number
Elastic fibers	Ordinary	Dominant in microfibrils (Fig. 4)	Ordinary	Dominant in microfibrils
Glycosaminoglycans	Rods on collagen fibrils. Spots and filaments in the interfibrous spaces (Fig. 5a)	Long rods forming meshes around collagen fibrils. Many distinct spots and filaments (Fig. 5b)	Indistinct, thinner and shorter rods. Indistinct irregular shapes of interfibrous filaments and spots (Fig. 5c)	Shapes resembled the betamethasone group but they appeared distinct (Fig. 5d)

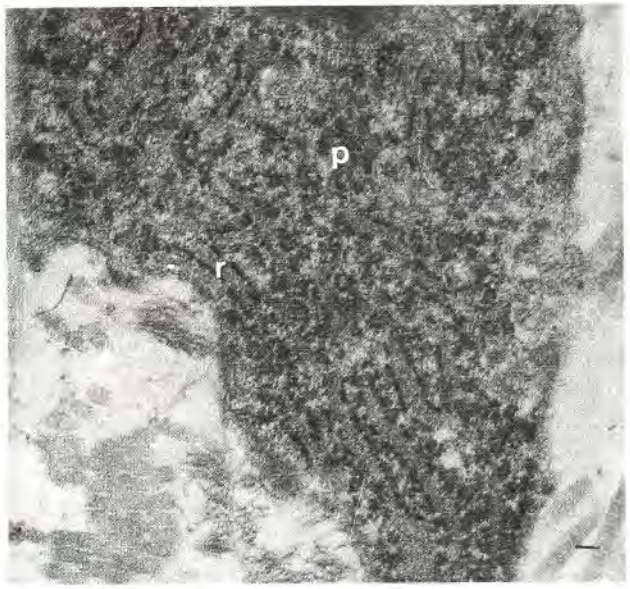

Fig. 2. The fibroblasts in the KH-1060-treated dermis contain granular endoplasmic reticulum (*r*) and polyribosomes (*p*). ×30,000. *Bar*: 0.1 μ.

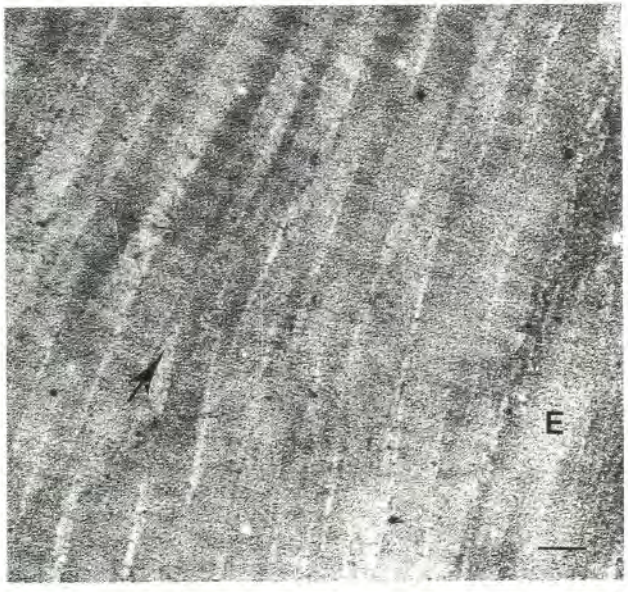

Fig. 3. Compact bundles of collagen fibrils and an elastic fiber (*E*). Twisted collagen fibrils (*arrows*). ×60,000. *Bar*: 0.1 μ.

collagen fibrils in the collagen bundles (Fig. 3). Both KH-1060 and betamethasone reduced the number of mast cells. Mast cells in the KH-1060-treated group contained normal mature granules and showed no extrusion of the granules (Fig. 1). These signs were remarkable, when compared with the other three groups. No differences were found in mast cell granules among these three groups. Glycosaminoglycan figures in the interfibrous space appeared distinct after KH-1060 treatment (Fig. 5b), compared with Fig. 5a). This effect was also noticed in the group treated with both KH-1060 and betamethasone (Fig. 5d, compared with Fig. 5c).

After immunochemical staining for type I collagen, gold particles were found exclusively in collagen fibrils (Fig. 6). No gold particles were present in any other tissue components of the dermis. After the control staining, no gold particles could

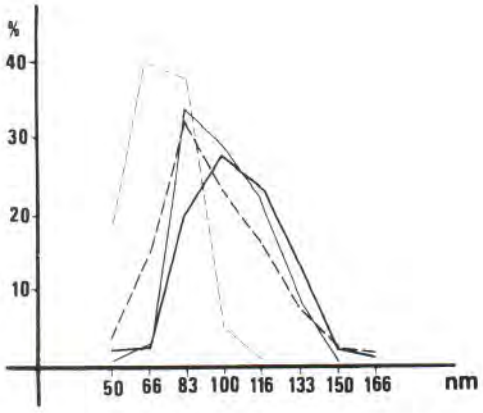

Fig. 4. Distribution of thickness and population of collagen fibrils per unit area (1 μm^2) of the collagen bundles ($n = 10$ for each group). Isopropanol: —; KH-1060: **—**; Betamethasone: —; Betamethasone and KH-1060; **- -**.

be demonstrated in the sections. The number of gold particles in the collagen fibrils was not changed by KH-1060 treatment (Table IV).

DISCUSSION

The present study demonstrates that KH-1060 stimulates collagen fibril formation. The ultrastructure of fibroblasts in this study and the increased uptake of H^3-proline after KH-1060 treatment (4) suggest stimulation of the formation of collagen fibrils. Collagen fibrils were thickened in the KH-1060-treated group, while collagen fibrils were thinner in the betamethasone-treated group. KH-1060 was effective in thickening the collagen fibrils in the betamethasone-treated dermis, so that the thin collagen fibrils developed to normal thickness. It has been observed that glycocorticoid produces thin collagen fibrils in the dermis of normal mice (8).

There was no increase of type I collagen in collagen fibrils after KH-1060 treatment. This implies that the effect of KH-1060 might be an increase of type III collagen content. Previous studies by immunoelectron microscopy have demonstrated that, in granulation tissue of leg ulcer (10) and in scleroderma (11), the ratio of type I collagen to type III collagen in collagen fibrils was low in the early phases of fibrosis; later on the ratio increased to close to the normal value of 2.5. Therefore, in human fibrosis, secretion of collagen could be initiated by type III collagen and followed by type I collagen. KH-1060 presumably stimulated fibroblasts to produce type III collagen. However, the problem on KH-1060 stimulation for production of collagen types I and III remains unsolved, as the 4-week period during which the experiment was conducted was not long enough to study time-dependent changes in the ratio of type I to type III collagen. In addition, goat antibody to murine collagen type III was not available.

Collagenase production is another function of epithelial cells and fibroblasts. However, since no ultrastructural signs of collagen degradation (9) were found, this function did not seem to be stimulated by KH-1060. These findings were in contrast to retinoid, which reduced collagen formation by disturbing protein metabolism of fibroblasts (13). The twisted shapes of collagen fibrils did not represent an effect of KH-1060. Apparently, twisted collagen fibrils are common in

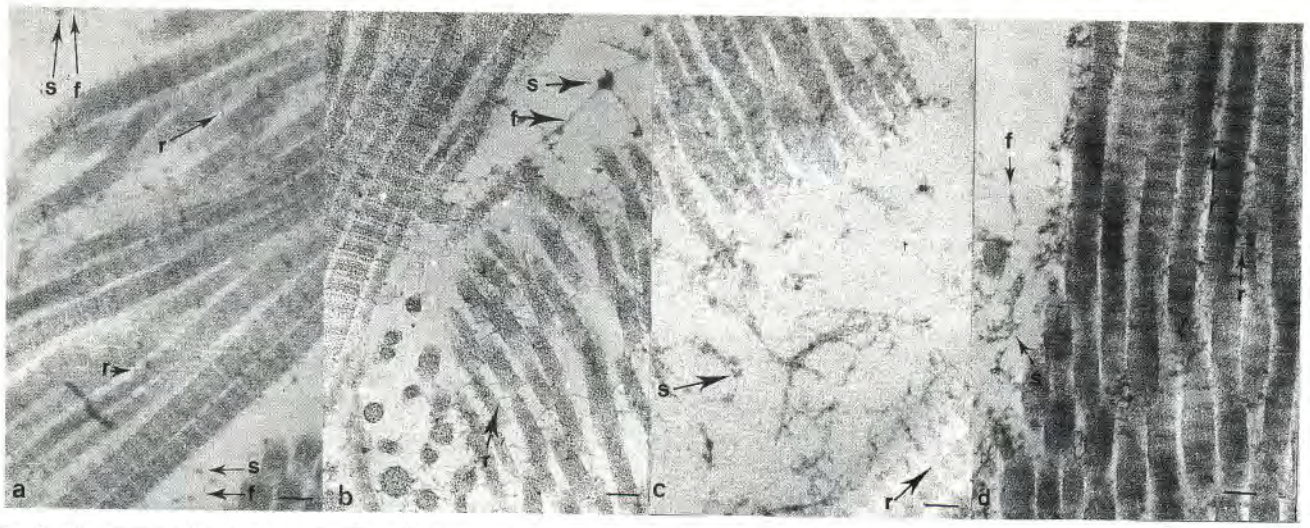

Fig. 5. Glycosaminoglycan figures in the vehicle-treated group (*a*) appear as rods on the surface of the collagen fibrils (*arrow with r*) and filaments (*arrow with f*) and spots (*arrow with s*) in the spaces between the adjacent bundles of collagen fibrils. When *a* is compared with *b–d*, the KH-1060 treatment reveals long rods and filaments with spots (*b*). The betamethasone-treated group shows thin, short rods, irregularly shaped interfibrous spots and filaments (*c*). The concomitant treatment makes these figures distinct (*d*). ×60,000. *Bar*: 0.1 μ.

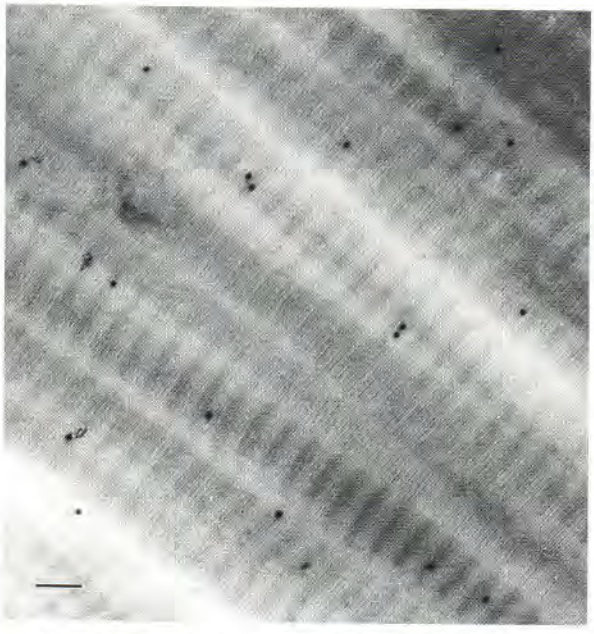

Fig. 6. Type I collagen in the collagen fibrils after KH-1060 treatment. Collagen type I is demonstrated by 10-nm gold particles. Gold particles appear in relation to dense wide cross-bands of collagen fibrils. The comparison among the experimental groups appears in Table IV. ×60,000. *Bar*: 0.1 μ.

the dermis of hairless mice (14). In human dermis, this type of collagen fibril indicates an inherited disorder of collagen metabolism (15). It is suggested that inherited changes in collagen metabolism are present in dermis of hairless mice.

Bundles of elastic microfibrils in human dermis are usually found in young individuals. These microfibrils disappear with age (16). The same process could take place in mice. KH-1060 perhaps increased the formation of elastic microfibrils.

When mast cells in the KH-1060-treated group were compared to those in the vehicle group, KH-1060 was more effective in the production of normal mature granules and in the suppression of granule extrusion. It has been demonstrated that, in human as well as in mouse mast cells, degranulation occurs by two modes: extrusion of non-disintegrated granules and secretion of disintegrated granular content, leaving honeycomb-like structures in the cytoplasm (17). Corticosteroid produced numerous abnormal granules and stimulated degranulation (18). It appears that, if mast cells are first treated with betamethasone, KH-1060 no longer affects them.

Vacuolation in the mitochondria is a common phenomenon in KH-1060 treatment. This phenomenon was also found in the keratinocytes after KH-1060 treatment (7).

Glycosaminoglycans in dermis exhibit three characteristics in electron microscopy: branched filaments, round spots in interfibrous spaces and rods joined to cross bands of collagen fibrils (19). These figures vary in normal tissues and also in diseased conditions; i.e. Walton's jelly and myxedematous skin revealed more distinct spots and rods, while normal dermis presented more filamentous shapes (19, 20). Topical application of budesonide produced irregularly shaped indistinct thin filaments in the interfibrous space (8), similar to those seen in the betamethasone-treated group of this study. KH-1060 increased the density of glycosaminoglycan figures, though the shapes of the figures were not influenced. These findings were consistent with an increased uptake of ^{35}S-sulphate by KH-1060 (4, 5).

ACKNOWLEDGEMENTS

The animal experiments in this study were carried out by Dr Robert Gniadecki in the laboratories of LEO Pharmaceutical Products, Denmark.

This study was supported by grants from the Danish Rheumatism Foundation, Gerda and Aage Hænsch's Foundation and Terd & Ellen Hindsgaard's Foundation.

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