The Lovastatin-Treated Rodent: A New Model of Barrier Disruption and Epidermal Hyperplasia

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Recent studies have linked epidermal cholesterol synthesis with maintenance of the permeability barrier. To assess directly the importance of cholesterol synthesis, we applied lovastatin, a potent inhibitor of cholesterol synthesis, to hairless mouse skin. Transepidermal water loss (TEWL) began to increase after four to six daily applications. Co-application of cholesterol blocked the expected increase in TEWL, demonstrating the importance of cholesterol for development of the lesion. The histology of lovastatin-treated skin revealed epidermal hyperplasia, accompanied by accelerated DNA synthesis. Whereas cholesterol synthesis initially was reduced in lovastatin-treated epidermis, with further treatment cholesterol synthesis normalized, while fatty acid synthesis accelerated greatly. Although the total free sterol content of lovastatin-treated epidermis remained normal, the fatty acid content increased coincident with barrier disruption. Finally, morphologic abnormalities of both lamellar body structure and their deposited, intercellular contents occurred coincident with the emerging biochemical abnormalities. Thus, the abnormal barrier function in this model can be ascribed to an initial inhibition of epidermal sterol synthesis followed by an alteration in cholesterol and fatty acid synthesis, leading to an imbalance in stratum corneum lipid composition and abnormal membrane bilayer structure. J Invest Dermatol 96:201–209, 1991

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bundant evidence supports a role for stratum corneum lipids in mammalian cutaneous barrier function (reviewed in [1–3]). These lipids contain approximately equal quantities of ceramides, cholesterol, and free fatty acids, as well as lesser amounts of non-polar lipids and cholesterol sulfate, arranged as broad membrane bilayers in the stratum corneum interstices [2,4–6]. Although various studies support a role for sphingolipids and fatty acids in barrier function, the evidence for the importance of cholesterol is particularly persuasive [7–10]. First, a rapid increase in sterol genesis follows various types of barrier perturbation [7–9]; second, sterol genesis returns to normal as barrier function normalizes [7,9]; third, the burst in sterol synthesis does not occur when experimentally perturbed skin is occluded with a vapor-impermeable occlusive membrane [7–10]; fourth, both the total activity and activation state of HMG CoA reductase, the rate-limiting enzyme in cholesterol synthesis [11], are modulated in response to barrier requirements [12]. Finally, in recent studies we found that topical applications of lovastatin, a competitive inhibitor of HMG CoA reductase, interfered with the return of barrier function to normal following experimental perturbation [13].

As a further test of the importance of cholesterol synthesis for barrier function, in the present study we assessed the effects of daily applications of topical lovastatin on barrier function in intact skin. Lovastatin (mevinolin, Mevacor) is a potent competitive inhibitor of HMG CoA reductase [14]. We report here that repeated topical applications of lovastatin to normal hairless mouse skin lead to a perturbation of barrier function. Although this barrier abnormality is associated acutely with selective inhibition of cutaneous sterol genesis, a compensatory increase in HMG CoA reductase activity leads to a normalization of epidermal sterol synthesis and content. Rather than reduced sterol content, the barrier abnormality is associated with a disproportionate increase in fatty acid synthesis leading to altered sterol:acylceramide ratios. The abnormal lipid biosynthetic activity produces distinctive ultrastructural abnormalities in the contents of epidermal lamellar bodies, the putative organelle responsible for the formation of the permeability barrier. Finally, the lovastatin model is associated with epidermal hyperplasia and increased DNA synthesis, which could either represent an abortive attempt at barrier repair, or an additional cause of the barrier abnormality.

METHODS AND MATERIALS

Materials Hairless male mice (h/h), 8–12 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME). They were fed Purina mouse diet and water ad libitum. Their age ranged between 2–3 months at the time of study. Acetone was purchased from

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Fishler Scientific (Fairlane, NJ). [1-14C]-Acetate, sodium salt (50–55 µCi/mM), [7n-3H]-cholesterol (31 Ci/mM), [9,10-(n-3H)]oleic acid (2–10 Ci/mM), [14C-3-hydroxy-3-methylglutaryl-Coenzyme A (DL-3-[3H]-glutaryl-3-[14C]) (HMG CoA, 54.2 mCi/mM), and 3H-mevalonolactone, RS-[5-3Hn] (PH-MVA, 38.8 Ci/mM) were purchased from New England Nuclear (Boston, MA). Desmosterol, 7-dehydrocholesterol, and cholecalciferol were purchased from Research Plus (Bayonne, NJ). Ruthenium tetroxide was obtained from Polysciences Inc. (Warrington, PA). Cholesterol, mevalonate, ethylenediaminetetraacetic acid (EDTA), glucose-6-phosphate, glucose-6-phosphate dehydrogenase (440 units/mg protein), NADP, and diithiothreitol (DTT) were purchased from Sigma Chemical Company. Anion exchange silica gel (AG 1-x8, formate form, 200–400 mesh) was purchased from Bio-Rad Laboratories (Richmond, CA). The lovastatin was a gift from Dr. A. Alberts, Merck, Sharpe & Dohme, Inc., Rathway, NJ.

**Experimental Protocols**

The lovastatin solution, solubilized in propylene glycol: ethanol (7:3 vols, 25 mg/mL), was applied once daily (30 µl) to an approximately 2-cm² area on one flank of groups of five to nine mice. Vehicle alone was applied to one flank of a separate group of animals. In both groups, the contralateral, untreated side served as the control. Transepidermal water loss (TEWL) was measured with an electrolytic water analyzer (Meeco, Warrington, PA) [7–9], on both the treated and untreated sites daily or other day, immediately prior to the next topical application. The raw data, obtained in ppm/0.5 cm²/h over background, were converted to mg/cm²/h according to the formula: \( J = 6 \times (18 \, P/22.4A) \times 10^{-3} \) (P = increase over background, A = area of skin).

In additional studies, lovastatin was applied once daily unilaterally as described above, whereas topical applications of either cholesterol 25 mg/mL or vehicle were administered three times daily. Desoximetasone (Topicort) cream (0.1 mg/application) or the vehicle for the steroid was applied three times daily to lovastatin-treated animals in other experiments. TEWL was measured daily for up to 8 d over treated and untreated sides. At the end of experiments, skin samples were obtained for biochemical and microscopical studies (see below).

**Epidermal Lipid Analysis**

Hairless mice were sacrificed before treatment, and 1, 5, and 8 d after treatment with lovastatin. Epidermis was obtained from the whole skin of hairless mice by exposure to dry heat, 60°C for 60 sec, a procedure that separates epidermis as an intact sheet from the underlying dermis [15]. Our standard trypsinization method for the isolation of intact stratum corneum sheets [15] could not be applied to lovastatin-treated skin because of its poor cohesion, i.e., excessive scaliness. Pooled epidermal sheets were extracted by the method of Bligh-Dyer [16], dried, and weighed. For gravimetric quantitation of pooled samples, 2-mg samples of the lipid extract and standards were applied to pre-cleaned thin-layer chromatography (TLC) plates (high-performance TLC silica gel 60; Merck, Darmstadt, FRG), and fractionated sequentially in neutral lipid (petroleum ether: diethyl ether: glacial acetic acid, 80:20:1 vols) and sphingolipid (chloroform: methanol: water, 90:10:1 vols) solvent systems, as described previously [15,17]. The major species were identified under UVA fluorescence after spraying with 0.25% aqueous 8-anilino-1-naphthalene sulfonic acid. Free sterols were fractionated into cholesterol, 7-dehydrocholesterol, lathosterol, and lanosterol sub-species, using a normal-phase high-pressure liquid chromatography system [18].

**Lipid Synthesis**

Twenty-four hours and 10 d following daily treatments with either lovastatin or vehicle, the full-thickness skin samples were removed and placed dermis-side-downward in 2 ml of a Ca³⁺–Mg²⁺-free phosphate-buffered saline (pH 7.4) containing 40 µCi of [14C]-acetate and 10 nM EDTA. The full-thickness skin samples were incubated for 2 h at 37°C and then the epidermis was separated manually from the dermis. Saponifiable lipids and cholesterol were obtained and quantitated as described previously [19].

**HMG CoA Reductase Assay**

After mice were killed by cervical dislocation, the tissue and, in some experiments, the liver, was excised. The epidermis of treated and control skin samples was separated from the dermis by incubating in 10 mM EDTA in Dulbecco's Ca⁺⁺- and Mg⁺⁺-free, phosphate-buffered saline 37°C for 40 min, and microsomes were prepared from the epidermal samples, as recently described [12], except that following the 10,000 x g centrifugation, the supernatant was dialyzed for 3 d against the homogenization buffer using a 6,000–8,000 dalton cutoff membrane to remove residual lovastatin [20]. The dialyzed solution then was centrifuged at 100,000 x g for 60 min, the supernatant was removed, and the microsomal pellets were stored at -70°C until assayed. In control microsomes, dialysis did not result in a loss of HMG CoA reductase activity. HMG CoA reductase activity was determined as described previously by this laboratory [21], and was expressed as nmol mevalonate synthesized/mg protein/min. Protein was determined with a Bio-Rad Laboratories (Richmond, CA) protein assay dye reagent [22].

**Light and Electron Microscopy**

At various time points following lovastatin application, skin biopsies were taken from treated and untreated sides of both lovastatin and vehicle-treated animals for light and electron microscopy. For light microscopy, biopsies were fixed in phosphate-buffered formaldehyde, embedded in paraffin, and 6-µm sections were stained with hematoxylin and eosin. Parallel unfixed samples were snap-frozen in liquid nitrogen, and stored at -70°C until sectioned (6 µm) for fluorescence microscopy. Nile red (100 µg/mL, 75% glycerol) was applied to sections to detect the distribution of neutral lipids in lovastatin- vs. vehicle-treated tissues [10]. Stained sections were examined in a Leitz Ortholux II microscope equipped with epifluorescence at 450 nm excitation and 500 nm emission wavelengths, respectively.

Samples for electron microscopy were minced to 0.5 mm and fixed in half-strength Karnovsky's fixative overnight, washed in cacodylate buffer, and post-fixed in either 0.2% ruthenium or 2% osmium tetroxide containing 1.5% potassium ferrocyanide. Previously published protocols for ruthenium fixation [23] were modified both to optimize penetration of fixative and to minimize toxicity [24]. Briefly, aldehyde-fixed tissues were cryoprotected in a cryoprotectant buffer containing 7% sucrose, 10% glycerol, and 1% DMSO in 0.1 M cacodylate buffer, pH 7.4, sectioned in a cryostat...
(50 μm), and then post-fixed in 0.2% ruthenium tetroxide containing 1.5% potassium ferrocyanide in 0.1 M cacodylate buffer for 30 min in the dark. After fixation, all samples were dehydrated in graded ethanol solutions, and embedded in an Epon-epoxy mixture [25]. Six hundred to 800-nm sections were examined in a Zeiss 10A electron microscope operated at 60 kV either unstained or after brief additional contrasting with lead citrate.

Statistical significances were determined using the Student t test.

RESULTS

Cutaneous Lipid Synthesis in Lovastatin-Versus Vehicle-Treated Tissues As described in detail elsewhere, a single topical application of lovastatin produced a significant decrease in epidermal cholesterol synthesis, with no change in fatty acid synthesis at 2 and 5 h [13]. In Fig 1, the effects of topical lovastatin on the incorporation of acetate into cholesterol and fatty acids are shown 24 h after a single application of lovastatin, and after ten daily applications of the drug. At 5 h, cholesterol synthesis was significantly suppressed, whereas fatty acid synthesis was unchanged [13]. By 24 h, epidermal cholesterol synthesis remained significantly decreased (~52%; p < 0.05), whereas fatty acid synthesis was significantly increased (+61%; p < 0.05). At this timepoint, cholesterol synthesis also was significantly decreased in the dermis, with no changes in fatty acid synthesis. By 10 d, the rate of epidermal and dermal cholesterol synthesis returned to levels comparable to or greater than those in the vehicle-treated sample, while epidermal fatty acid synthesis increased even further (+277%; p < 0.001). These results demonstrate that topical applications of lovastatin initially inhibit epidermal sterologenesis, followed by normalization at later time points. In contrast, fatty acid synthesis is accelerated by day one, and continues to be elevated at 10 d.

Lipid Content of Lovastatin-Versus Vehicle-Treated Epidermis We next assessed the effects of repeated lovastatin applications on epidermal lipid content in lovastatin-versus vehicle-treated animals. As noted above, it was not possible to isolate intact stratum corneum from lovastatin-treated animals. However, because about 50% of the lipid content of the epidermis derives from the stratum corneum [15], major changes in epidermal lipid composition should be reflective of alterations in the stratum corneum. Immediately before lovastatin treatment and 1 d after the initial application, both the free sterol and the fatty acid:total lipid ratios were comparable (Table I). By 5 d, however, coincident with an initial rise in TEWL, the fatty acid content increased by 73% (p < 0.01) (Table I). By 8 d, the increase in fatty acid content became even more apparent, representing over 20% of the lipids in lovastatin-treated epidermis (Table I Fig 3). These trends hold even when corrected for the putative sebaceous gland origin of sterol esters (Table I, parentheses). In contrast, the free sterol and sphingolipid content did not change substantially. Moreover, further fractionation of the free sterols by high-performance HPLC revealed no significant differences in the distribution of cholesterol, 7-dehydrocholesterol, lanosterol, and lathosterol in lovastatin-versus vehicle-treated epidermis (data not shown).

In addition to the changes in free fatty acids noted above, triglyceride content increased markedly at d 1, a change that persisted at day 5 and began to decrease at day 8 (Table I) (Fig 3). The lipid weight/mg dry weight of lovastatin-treated versus untreated (0 time) epidermal samples did not differ significantly (weight data not shown). Because triglycerides are largely intracellular and/or of sebaceous gland origin, and sterol esters derive largely from sebaceous glands [26], the most noteworthy finding appears to be the alterations in free fatty acid content in these animals. These studies show that chronic treatment with lovastatin produces a persistent

Table I. Lipid Composition in Lovastatin-Treated Epidermis* (distribution minus sterol esters)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Day 0 (n = 4)</th>
<th>Day 1 (n = 5)</th>
<th>Day 5 (n = 5)</th>
<th>Day 8 (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterol Esters</td>
<td>60.3 ± 2.9</td>
<td>50.6 ± 6.4</td>
<td>39.2 ± 1.3*</td>
<td>29.9 ± 5.2*</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>(20.2) 8.0 ± 1.6</td>
<td>(28.4) 14.1 ± 3.0*</td>
<td>(23.7) 14.4 ± 3.2*</td>
<td>(17.6) 12.3 ± 2.5*</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>(12.9) 5.1 ± 0.2</td>
<td>(11.7) 5.8 ± 0.8</td>
<td>(14.5) 8.8 ± 0.6*</td>
<td>(30.1) 21.1 ± 6.9*</td>
</tr>
<tr>
<td>Free sterols</td>
<td>(36.6) 14.5 ± 0.4</td>
<td>(31.4) 15.5 ± 2.3</td>
<td>(35.9) 21.8 ± 1.3*</td>
<td>(26.6) 18.6 ± 7.9</td>
</tr>
<tr>
<td>Sphingolipids</td>
<td>(30.3) 12.0 ± 2.1</td>
<td>(28.3) 14.0 ± 7.0</td>
<td>(30.0) 15.8 ± 2.0</td>
<td>(25.7) 18.0 ± 6.4</td>
</tr>
<tr>
<td>Total</td>
<td>(100.0) 100.0</td>
<td>(100.0) 100.0</td>
<td>(100.0) 100.0</td>
<td>(100.0) 100.0</td>
</tr>
</tbody>
</table>

* Lipid distribution in lovastatin-treated murine epidermis (0–8 d; percent distribution = mean ± SE of all samples at each time point). TEWL increased first at d 5, and greatly between 6 and 7 d in this experiment. Note the progressive increase in the proportion of free fatty acids and triglycerides at 5 and 8 d, while free sterols and total sphingolipids do not change significantly. Because most of the sterol esters in hairless mouse epidermis may be of sebaceous gland origin [26], the data are also shown corrected for sebaceous contribution (in parentheses). The same trends are seen ± sterol esters.

<sup>1</sup> p < 0.05 versus 0 time.
increase in fatty acid content, which coincides with the appearance of altered barrier function (see below).

**Epidermal Barrier Function and Macroscopic Effects of Lovastatin Applications** Although repeated daily applications of lovastatin produced no changes in rates of transepidermal water loss (TEWL) on the treated side for the first few days, TEWL rates began to increase after 5–7 d (Table II). Whereas the untreated side initially showed no change, it too increased dramatically 1–2 d after the treated side began to respond (Table II). After several days, TEWL rates were comparable on both the treated and untreated side. In contrast, the vehicle did not produce significant changes in TEWL, even after repeated applications.

At the same time that the animals exhibited elevated rates of TEWL, they developed generalized erythema and scaling, more pronounced initially on the treated side (Fig 4). But, after several days, the scaling and erythema were equally pronounced on both the treated and untreated sites. These results show that repeated applications of lovastatin to intact skin produce a progressive abnormality in barrier function that affects treated sites initially, spreading to untreated sites on the same animal after a 1–2 d delay.

When cholesterol, the final product of the sterol biosynthetic pathway, was co-applied with lovastatin, TEWL rates increased at a much slower rate than they did in animals treated with lovastatin alone (Fig 5). Moreover, coapplications of cholesterol blunted the appearance of scaling and erythema (Fig 4). When lovastatin-treated animals were treated simultaneously with a potent topical steroid, desoximetasone, the defect in barrier function occurred at the same rate as in vehicle-treated animals (lovastatin + steroid, 1.50 ± 0.47, versus lovastatin + cream vehicle for steroid, 1.66 ± 0.35 mg/cm²/h; n = 5). Moreover, the extent of erythema and scaling was equally prominent in both groups. These results suggest first, that cholesterol is the product of HMG CoA reductase that is required to maintain normal barrier function, and second, that non-specific inflammation in itself is not a likely explanation for the lovastatin-induced effect.

**Histology and Cell Kinetic Parameters in Lovastatin-Versus Vehicle-Treated Epidermis** (Fig 6) Light microscopy of lovastatin-treated epidermis displayed several striking alterations (Fig 6B). In contrast to vehicle-treated samples (Fig 6A), after several days a marked thickening of the viable cell layers (acanthosis and

<table>
<thead>
<tr>
<th>Day</th>
<th>Lovastatin Side</th>
<th>Untreated Side</th>
<th>Vehicle Side</th>
<th>Untreated Side</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.176 ± 0.028</td>
<td>0.170 ± 0.024</td>
<td>0.118 ± 0.009</td>
<td>0.110 ± 0.011</td>
</tr>
<tr>
<td>2</td>
<td>0.176 ± 0.020</td>
<td>0.176 ± 0.018</td>
<td>0.123 ± 0.016</td>
<td>0.104 ± 0.005</td>
</tr>
<tr>
<td>3</td>
<td>0.376 ± 0.097</td>
<td>0.168 ± 0.016</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>0.619 ± 0.094</td>
<td>0.326 ± 0.070</td>
<td>0.150 ± 0.035</td>
<td>0.120 ± 0.026</td>
</tr>
<tr>
<td>5</td>
<td>2.086 ± 0.51</td>
<td>1.014 ± 0.32</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>&gt; 5.0</td>
<td>&gt; 5.0</td>
<td>0.197 ± 0.057</td>
<td>0.208 ± 0.036</td>
</tr>
<tr>
<td>7</td>
<td>&gt; 5.0</td>
<td>&gt; 5.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>&gt; 5.0</td>
<td>&gt; 5.0</td>
<td>0.198 ± 0.041</td>
<td>0.164 ± 0.021</td>
</tr>
</tbody>
</table>

* Changes in barrier function on the treated and untreated sides of lovastatin versus vehicle-treated animals. After 5 d, both treated and untreated side exhibit water loss rates in excess of 5 mg/cm²/h (accurate measurements were not possible due to the extensive scaling, which interferes with contact of the probe with the skin). The vehicle also produced minor perturbations in barrier function. Results are presented as mean ± SEM.

* ND, Not determined.

Figure 3. Thin-layer chromatograms of lipid extracts from lovastatin (L)-versus vehicle (V)-treated epidermis after 8 d treatment. Note that the free sterol (FS) band appears slightly more prominent, whereas the free fatty acids (FFA), triglycerides (TG), and sterol esters (SE) appear much more prominent in lovastatin- than in vehicle-treated samples. The same quantity of lipid was applied to each lane. S, standards.

Figure 4. Appearance of hairless mice after four daily applications of lovastatin (L, 25 mg/ml) or lovastatin + cholesterol (L + C, 25 mg/ml), to the right side. The contralateral side was left untreated (U) in each case. In animals treated with L alone, note generalized erythema and scaling, more pronounced at the sites ofLovastatin treatment. In contrast, co-administration of cholesterol largely prevents the appearance of scaling.
These results are consistent with a time delay in DNA synthesis on the untreated versus treated side that correlates with the delay in barrier disruption on the untreated side (cf Table II). These results show that lovastatin treatment results in stimulation of epidermal DNA synthesis leading to marked hyperplasia.

**Histochecmistry and Ultrastructure of Lovastatin-Treated Epidermis** Frozen sections of lovastatin-treated (6–8 d) epidermis, stained with nile red, displayed brightly fluorescent lipid droplets within the cytosol of cornified cells on both treated and untreated sides, but not in vehicle-treated controls (Fig 7; vehicle side not shown). In contrast, the overall fluorescence intensity of lovastatin versus control stratum corneum stained with filipin, a specific indicator of 3β-hydroxy steroids, did not differ (data not shown). These results are consistent with the biochemical studies (cf Table I; i.e., that lovastatin treatment results in a generation of excess free fatty acids.

Electron microscopy of ruthenium tetroxide-fixed, lovastatin-treated sites revealed a striking abnormality in both lamellar body size and internal structure (Fig 8). In normal epidermis, lamellar bodies are ellipsoidal, measuring 0.25–0.5 μm in short and long axis, respectively (Fig 8, inset). In contrast, lamellar bodies in lovastatin-treated epidermis were much larger than normal (>1 μm), and mostly spheroidal rather than ellipsoidal. In addition, they displayed abnormal internal structure (Fig 8): instead of parallel arrays of discs, the lamellar contents often appeared elongated, distorted, or fragmented, and discs were often displaced peripherally by electron-lucent droplets within the organelle. In many sites, these abnormal lamellar bodies were secreted into the intercellular spaces (Fig 8). But often, lamellar body contents were retained within cornified cells resulting in the retention of considerable lamellar body-derived lipids (Fig 8). These results demonstrate the deposition of abnormal lamellar body contents in the intercellular spaces of the stratum corneum, and, as in the nile red-stained frozen sections, considerable retention of lamellar body-derived lipids within cornified cells (cf Fig 7).

**DISCUSSION**

Because of their localization to intercellular domains, their organization into lamellar bilayers, and their relatively hydrophobic composition, stratum corneum lipids are considered the principal regula-

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**Figure 5.** Effect of co-administration of cholesterol on lovastatin-induced barrier disruption. Changes in barrier function are shown in animals treated with lovastatin (solid diamonds) versus lovastatin plus cholesterol (open squares). Note that co-administration of cholesterol significantly slows the development of abnormal barrier function at days 3–6. Results are mean ± SEM.

**Figure 6.** Histology of vehicle-treated (a) and lovastatin-treated (b) skin after 8 d treatment. The vehicle-treated skin (a) does not differ from the untreated controls (not shown), revealing 2–3 nucleated epidermal (E) cell layers, surrounded by a thin, compact stratum corneum (SC). No inflammatory cells are present in the dermis (D). In contrast, both the treated and the untreated skin from lovastatin-treated animals reveal an increase in the number of epidermal cell layers, focal hyperkeratosis, increased mitotic figures (arrows), and a sparse lymphohistiocytic infiltrate in the dermis (b; untreated side of lovastatin animals not shown). Hematoxylin + eosin; a and b, magnification ×1,500.
Table III. \(^3\H\text{-Thymidine Incorporation and DNA Content in Lovastatin-Treated Epidermis}\)

<table>
<thead>
<tr>
<th>Condition</th>
<th>TEWL (mg/cm(^2)/h)</th>
<th>(^3\H\text{-Thymidine Incorporation (% Change)}) (dpm/cm(^2)/h)</th>
<th>DNA Content (% Change) (g/cm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lovastatin-treated side (n = 3)</td>
<td>3.95 ± 0.62</td>
<td>8,456 ± 811* (164%)</td>
<td>63±6 (113%)</td>
</tr>
<tr>
<td>Untreated side, lovastatin animal (n = 3)</td>
<td>1.06 ± 0.27</td>
<td>8,492 ± 519* (163%)</td>
<td>44±8 (163%)</td>
</tr>
<tr>
<td>Vehicle-treated control animal (n = 3)</td>
<td>0.116 ± 0.019</td>
<td>5,219 ± 442 (--)</td>
<td>27±4 (--)</td>
</tr>
</tbody>
</table>

* p < 0.01 versus vehicle-treated controls.

Barrier function, DNA synthesis, and DNA content in lovastatin-treated and vehicle-treated (treated and untreated) animals. After 7 d of lovastatin treatment, there was the same increase in \(^3\H\text{-thymidine incorporation on both treated and untreated side of lovastatin-treated animals. In contrast, the increase in DNA content was much higher on the treated than on the untreated side. Results are presented as mean ± SEM.

Tors of the epidermal permeability barrier [1,2]. Such criteria, however, provide only indirect evidence for the role of lipids in the barrier. To obtain more direct evidence for the role of specific lipids in barrier function, we have recently assessed the lipid metabolic response to experimental barrier perturbation, and in various model systems, have shown that the synthesis of both epidermal sterols and fatty acids are regulated by barrier requirements [3,7-10]. To assess more directly the role of sterols for barrier function, we applied lovastatin, a competitive inhibitor of HMG CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis [11], to hairless mouse skin in two types of studies. First, in recent studies we showed that topical lovastatin delays the recovery of barrier function following solvent-induced barrier disruption [13]. Second, as reported here, we found that repeated applications of lovastatin to intact skin results in a progressive defect in the epidermal permeability barrier.

Whereas the genesis of the barrier abnormality in this chronic model must still be considered speculative, the lipid biosynthetic, lipid biochemical, and ultrastructural studies suggest that a specific sequence occurs that ultimately establishes the chronic barrier abnormality (Fig 9). Lovastatin application leads initially to an inhibition in HMG CoA reductase activity, which results in a selective decrease in cholesterol synthesis at 24 h (Table I). In fact, lovastatin inhibits epidermal sterol synthesis at even earlier time points (2 and 5 h) [13], as well as inhibiting sterol synthesis in cultured human epidermal cells [27,28]. Yet, despite the acute depression in chole-

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**Figure 7.** Nile red-stained frozen sections of lovastatin-treated skin, examined by fluorescence microscopy. Note neutral lipid-containing droplets in cytosol of cornified cells. Magnification X250. **Figure 8.** Electron micrograph of lovastatin-treated epidermis (day 7; TEWL > 500) at stratum corneum (SC)-stratum granulosum (SG) interface. Note abnormal size and content of lamellar bodies (LB), as well as presence of abnormal LB contents within intercellular spaces (arrows). Inset shows a normal lamellar body for comparison. Ruthenium tetroxide stained, magnification 75,000; inset, magnification X95,000.
cholesterol synthesis, establishment of the chronic barrier abnormality appears to be more complex than simple suppression of cholesterol synthesis. In fact, both cholesterol synthesis and free sterol content are normal by the time the barrier defect first occurs, presumably due to a compensatory increase in HMG CoA reductase activity. Such a compensatory response occurs in other systems, where a) feeding rats lovastatin results in an eventual increase in HMG CoA reductase activity in liver, small intestine, heart, and kidney [30,31]; and b) addition of HMG CoA reductase inhibitors to cells in culture likewise results in a striking increase in HMG CoA reductase activity (e.g., [31]).

In addition to the cholesterol generated by local epidermal synthesis, additional cholesterol may be imported from the circulation as a result of the increased LDL receptor expression that follows lovastatin treatment [32]. Although the epidermis normally expresses only small numbers of LDL receptors [33,34], in hyperplastic conditions such as psoriasis, additional LDL receptors are expressed [35]. Although epidermal LDL receptor expression has not been studied in lovastatin-treated animals, hyperplasia is a prominent feature of this model, and enhanced receptor expression could represent a second mechanism whereby epidermal cholesterol levels might eventually be normalized in these animals.

The lovastatin-induced lesion theoretically could be ascribed to non-specific inflammation or allergic contact dermatitis. Increased transdermal water loss and epidermal hyperplasia are both features of all types of eczematous dermatitis [36]. Yet, co-administration of cholesterol blocked development of the barrier abnormality, and cholesterol has no antiphlogistic properties. Moreover, co-administration of potent topical corticosteroids does not prevent expression of the barrier abnormality. Finally, lovastatin analogues that do not inhibit HMG CoA reductase produce no cutaneous abnormalities when applied topically (Schaede et al., in preparation). These findings make it highly unlikely that non-specific inflammation forms the basis of the lovastatin model.

If not due simply to decreased cholesterol synthesis, and if not ascribable to inflammation induced by topical lovastatin, why then does the barrier abnormality persist and even worsen in the face of normalized sterol production and content? An important clue may be the boost in fatty acid synthesis that occurs synchronously with suppression of cholesterol synthesis. Although one explanation for such increased fatty acid synthesis might be the lipid biosynthetic burst that occurs after all forms of cutaneous barrier disruption [9,10], this seems unlikely because fatty acid synthesis here increases prior to barrier disruption. The more likely explanation is provided by recent studies in cultured keratinocytes dosed with lovastatin [28] or other HMG CoA reductase inhibitors (cholesterol sulfate [37]), where sterol synthesis also is suppressed and, just as occurred in these in vivo studies, fatty acid synthesis increases in the cultured keratinocytes 12–24 h after drug exposure [28,37]. Such an increase in fatty acid synthesis following lovastatin treatment has been noted previously in liver [38] and lens [39]. Because fatty acid synthetic rates are disproportionately higher than those for sterols, this could result in the altered sterol : fatty acid ratios shown in Table 1. These biochemical changes are likely to be relevant for establishment of the barrier abnormality because they coincide with the appearance of abnormal barrier function. In the stratum corneum of all terrestrial mammals studied to date (porcine, murine, and human), the predominant lipid species comprise an approximately equimolar mixture of ceramides, free sterols, and free fatty acids [15,17,40–43]. Smaller amounts of other lipids also are present, which may originate from either epidermal [15] and/or pilosebaceous gland [44] sources. Thus, changes in free fatty acid content could provide an explanation for the structural abnormalities that we observed in the lamellar body-secretory system of lovastatin-treated animals, changes that may be responsible for the barrier defect. In normal epidermis, the lamellar body is enriched in sphingolipids and free sterols, but not in free fatty acids [45]. These species, along with free fatty acids, derived from hydrolysis of phospholipids [46,47], are the source of the secreted lipids that eventually organize into stratum corneum intercellular bilayers [1–6]. Although the number of lamellar bodies in lovastatin-treated epidermis appears to be normal, they are abnormal in shape and content, which may reflect an alteration in the free sterol : acyl lipid ratio, i.e., accumulation of free fatty acids, which, in turn, could lead to abnormal barrier function. Physical-chemical studies in model lipid systems support the notion that altered ratios of sterols and acyl lipids can result in abnormal membrane function [48,50]. Moreover, in several inherited and acquired disorders of sterol metabolism, alterations in sterol : fatty acid ratios are associated with abnormal barrier function and/or desquamation [49,51,52].

In addition, many of the lamellar bodies in lovastatin-treated epidermis do not appear to be secreted normally. As seen with the nile red staining and by electron microscopy, abundant lipid and some lamellar bodies are retained within the corneocyte cytosol. Because the intercellular delivery and subsequent organization of lamellar body-derived lipids into membrane bilayers are prerequisites for barrier integrity [1,2], such retained, intracellular lipid would not be available for barrier formation. This maldistribution also explains, in part, the apparent paradox of abnormal barrier function in the face of normal epidermal cholesterol content in these animals, i.e., the intercellular domains may be selectively lipid-depleted, whereas the tissue as a whole is replete. A similar situation pertains in several other disorders of cornification, where intracellular lipid droplets, a defective lamellar body delivery system, and a relatively normal lipid profile co-exist [49].

Yet, lovastatin treatment results not only in a progressive defect in barrier function, but also in epidermal hyperplasia. Although the hyperplasia per se could contribute to the barrier abnormality, as is presumed to occur in disorders such as essential fatty acid deficiency [53] and in certain chronic dermatoses [36], the hyperplasia also could represent an abortive attempt to repair the barrier, as suggested by recent studies in several models of barrier disruption [54].

Lovastatin not only influences the barrier directly over sites of topical application, but also produces a generalized defect in barrier integrity and desquamation. It is possible that the distant effects of lovastatin can be ascribed to systemic absorption, with redistribution of drug to distant sites, because hepatic HMG CoA reductase content was increased. Moreover, the suppression of sterol synthesis in the dermis, observed here and at earlier time points (Fig 1) [13], suggests that the drug absorbs into deeper tissues, which are in contact with the vascular spaces. However, systemic administration of lovastatin has not been reported to induce cutaneous abnormalities, perhaps due to the relatively low delivery of drug for the skin with systemic administration. Therefore, it is more likely that the contralateral cutaneous changes are due to diffusion of lovastatin laterally across the skin surface. A third possibility, i.e., uninten-
tional ingestion of drug, is not likely to be the explanation because animals were caged individually and in some experiments, wore collars to prevent licking. Further studies are required to determine the mechanism of these contratalateral alterations.

We would like to thank Dr. Sreekumar Pillai for performance of the free sterol fractionations, Arthur H. Moser for technical assistance, and William Chapman for excellence in preparation of the manuscript.

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