Inhibitors of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Induce Reductase Accumulation and Altered Lamellar Bodies in Rat Forestomach Keratinocytes

Irwin I. Singer, Douglas W. Kawka, Solomon Scott, Philip Bailey, Michelle W. Kloss, James Majka, and James S. MacDonald

Lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and a potent hypocholesterolemic agent, induces a hyperplastic thickening of the rat forestomach mucosa after oral administration of its active form, a hydroxyacid. We studied the effects of lovastatin on the intracellular accumulation of HMG-CoA reductase immunostaining and the accompanying morphological changes in rat forestomach keratinocytes by immunofluorescence microscopy and transmission electron microscopy (TEM). Administration of lovastatin hydroxyacid induced increases in HMG-CoA reductase levels within forestomach keratinocytes that were dose and time dependent and reversible. The adjacent glandular stomach epithelium did not exhibit induction of reductase. A pharmacologically inactive epimer of lovastatin hydroxyacid did not increase keratinocyte reductase accumulation, and lovastatin lactone induced minimal forestomach reductase. TEM of forestomachs from rats given lovastatin hydroxyacid demonstrated profound alterations in epidermal lamellar bodies (organelles that transport lipids and steroids to the intercellular spaces of the stratum corneum). Treated cells lacked internal lipid lamellae and failed to secrete sheets of lipid material into the intercellular spaces of the stratum corneum. We hypothesize that sustained inhibition of HMG-CoA reductase in rat forestomach keratinocytes induces accumulation of HMG-CoA reductase and hyperplasia by inhibiting sterol synthesis, assembly of lamellar bodies, and formation of intercellular lipid sheets. (Arteriosclerosis and Thrombosis 1991;11:1156–1165)
permeability barrier is apparently linked to the assembly of cholesterol and other lipids into continuous intercellular sheets of the epidermal stratum corneum.\textsuperscript{21-23} Formation of this barrier seemed to result from coalescence of lipid lamellae secreted from lamellar bodies within the uppermost cells of the stratum granulosum.\textsuperscript{21,23} Disruption of the cutaneous water permeability barrier with organic solvents induced epidermal cholesterologenesis, accompanied by synthesis and activation of HMG-CoA reductase.\textsuperscript{24}

The forestomach of rodents (equivalent to the non-glandular stomach) exhibits an epithelium histologically indistinguishable from the mammalian epidermis.\textsuperscript{25} It has been recently observed that various HMG-CoA reductase inhibitors induce reversible dose- and time-dependent hyperplastic (i.e., hyperkeratotic and acanthotic) alterations in the rodent forestomach and that the degree of change is correlated with the in vitro pharmacological potency of these compounds.\textsuperscript{26,27} In this study we show that HMG-CoA reductase inhibitors stimulate accumulation of HMG-CoA reductase IF and deformation of lamellar bodies and intercellular lipid sheets of the rat forestomach in a manner correlated with their pharmacological potency. These results provide a mechanistic explanation for the hyperplastic effects of HMG-CoA reductase inhibitors on the rodent forestomach.

**Methods**

**Experimental Animals**

Sprague-Dawley female rats (Cr1:CD[SD]BR) that were 5–7 weeks old and had a weight range of 75–200 g were obtained from Charles River Breeding Laboratories (Wilmington, Mass.). The rats were housed in wire mesh cages under normal illumination and were provided with food and water ad libitum. Animal care was performed in accordance with guidelines established by the National Institutes of Health and the US Department of Agriculture; our facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care. HMG-CoA reductase inhibitors synthesized at Merck Sharp & Dohme Laboratories were administered once daily in 0.5% methylcellulose (Dow Chemical Co.) at 5 ml/kg by gavage. Individual animals were identified with permanent markers. At the end of each experiment, the animals were killed 1–2 hours after the last dose of compound by exsanguination under ether or CO\textsubscript{2} anesthesia. Necropsies were performed, and the stomachs were examined for gross abnormalities.

**Microscopy**

At necropsy, portions of the forestomach, glandular stomach, esophagus, and liver were excised from all animals and frozen rapidly in OCT mounting medium (Miles Laboratories, Naperville, Ill.) with liquid N\textsubscript{2}-cooled Freon 22 for IF analysis. Unfixed 5-\textmu m cryostat sections were cut and immunostained with purified 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase immunofluorescence (IF) intensity of unfixed immunolabeled 5-\textmu m frozen sections of rat forestomach keratinocytes was assessed by the following values: 0=none, 1=very slight, 2=slight, 3=moderate, 4=intense, and 5=markedly intense. Results are expressed as the mean for each group (five each).

| Table 1. Effects of HMG-CoA Reductase Inhibitors on HMG-CoA Reductase Immunofluorescent Staining Intensity in Rat Forestomach Keratinocytes and Presence of Forestomach Pathology After 14 Days* |
|-----------------|-----------------|-----------------|
| Compound        | Mean IF intensity* | Pathology |
| Methyl cellulose, 0.5% (control) | 0.4 | None |
| MK-803 (lactone of lovastatin)\textsuperscript{†} | 1.4 | None |
| L-154,819 (lovastatin hydroxyacid)\textsuperscript{†} | 4.9 | Yes |
| L-665,465 (inactive epimer of L-154,819)\textsuperscript{†} | 0.6 | None |
| MK-733 (lactone of simvastatin)\textsuperscript{†} | 4.2 | Yes |

*3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase immunofluorescence (IF) intensity of unfixed immunolabeled 5-\textmu m frozen sections of rat forestomach keratinocytes was assessed by the following values: 0=none, 1=very slight, 2=slight, 3=moderate, 4=intense, and 5=markedly intense. Results are expressed as the mean for each group (five each).

†Compounds were dissolved in 0.5% methylcellulose and administered orally at 30 mg/kg/day.
tase IF was observed in the adjacent glandular stomach epithelium (Figure 1D), and esophageal epithelial reductase was not elevated (see below). Moderate reductase IF was also induced in periportal hepatocytes of the L-154,819-treated animals. Comparable increases in HMG-CoA reductase labeling and hyperplasia/acanthosis were seen in rat forestomach keratinocytes treated similarly with simvastatin (MK-733, a potent lactone analogue of lovastatin) (Table 1). In contrast to L-154,819 and simvastatin, a pharmacologically less active HMG-CoA reductase inhibitor such as lovastatin lactone (MK-803) did not induce hyperplasia/acanthosis or significant HMG-CoA reductase IF in forestomach keratinocytes (Figures 1E–G and Table 1). Similarly, L-665,465, a pharmacologically inactive epimer of L-154,819, did not induce reductase IF or histological alterations in the forestomach epithelium (Table 1). In contrast, the analogous esophageal epithelium exhibited slight to moderate HMG-CoA IF comparable to that of controls, regardless of the type of HMG-CoA-reductase inhibitor administered (Figures 1H and 1I and Table 2). Induc-
tion of HMG-CoA IF and hyperplasia in rat forestomach keratinocytes were therefore tissue and compound specific. These staining results were also immunospecific because IF staining of forestomach or esophageal keratinocytes was eliminated by preincubating the reductase antibodies with purified rat HMG-CoA reductase (Figures 1F and 11). Stimulation of maximum HMG-CoA reductase levels in forestomach keratinocytes was dosage and time dependent: it was first observed at 3 days with a dose of 75 mg/kg/day L-154,819 or at 7–14 days with a dose of 30 mg/kg/day of this compound. In each case, reductase induction and keratinocyte hyperplasia were reversed by suspending administration of the compound for 14 days (Table 3).

**Transmission Electron Microscopy of Forestomach Keratinocytes**

Control rat forestomach keratinocytes of the stratum granulosum had numerous lamellar bodies at their apexes (Figures 2A and 2C). Accumulation of intercellular lipid material was most pronounced at the interface of the stratum granulosum and stratum corneum (Figure 2A). At higher magnifications, the lipids of both lamellar bodies and the intercellular compartment appeared to be composed of structurally similar stacks of disks (Figures 2B and 2C). After administration of 75 mg/kg/day lovastatin hydroxyacid (L-154,819) for 14 days, the ultrastructure of the forestomach stratum granulosum and stratum corneum became profoundly altered (Figure 3). Intercellular material at the stratum granulosum–stratum corneum border and throughout the stratum corneum exhibited numerous gaps (Figure 3A) and completely lacked the customary sheets of lipid lamellae (Figures 3B and 3C). Instead, clusters of heterogeneously sized vesicles were scattered within this intercellular space. Lamellar bodies also displayed sparse amounts of lipid disks and alternatively contained heterogeneous spherules similar to the vesicles within the intercellular spaces (Figures 3B and 3C). These lamellar bodies were 65% larger than those of animals treated with a comparable dose of lovastatin lactone (MK-803), and they were much less numerous (59% decrease; Table 4). All rats with these features also displayed markedly intense levels of HMG-CoA reductase immunofluorescence (IF) intensity.

**FIGURE 1.** Immunofluorescence photomicrographs depicting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase distribution in frozen sections of forestomach or esophageal keratinocytes of rats treated with HMG-CoA reductase inhibitors. Rats were treated orally with 30 mg/kg/day lovastatin hydroxyacid (L-154,819) for 14 days (panels A–D). (Arrowheads throughout indicate high background in stratum corneum only.) Panel A: Hydroxyacid of lovastatin induces intense HMG-CoA reductase immunostaining in keratinocytes of forestomach (arrows). Panel B: Transverse section of L-154,819-treated forestomach shows induction of HMG-CoA reductase immunostaining in stratum granulosum (asterisks) but less labeling in the basal layer (b). Note that this epithelium contains more layers of keratinocytes (i.e., it is hyperplastic) relative to that of the lovastatin lactone–treated rat (panel E). Panel C: Incubating HMG-CoA reductase antibodies with purified HMG-CoA reductase inhibits specific reductase labeling in keratinocytes of forestomach epithelium (e), whereas stratum corneum exhibits high background staining (arrowheads). Panel D: Section of anterior stomach through its junction with the forestomach; nonglandular keratinocytes display intense HMG-CoA reductase labeling (arrows), whereas glandular epithelium (g) is totally unstained. Panel E: Forestomach epithelium (e) of rat given lovastatin lactone (MK-803) shows slight HMG-CoA reductase staining with high background in external keratin layer (arrowhead). Panel F: Section adjacent to that in panel E stained with HMG-CoA reductase immunoglobulin G pretreated with purified HMG-CoA reductase to block specific labeling; no HMG-CoA reductase staining is seen in epithelium (e) despite the high background of stratum corneum (arrowhead). Panel G: In control rats, forestomach epithelium (e) is only very slightly positive for HMG-CoA reductase. Panel H: After administration of lovastatin hydroxyacid, esophageal keratinocytes exhibit moderate HMG-CoA reductase staining (arrows), although stratum corneum is nonspecifically labeled (arrowheads). Panel I: Neighboring section of esophagus immunostained with HMG-CoA reductase antibodies preincubated with purified reductase exhibits elimination of specific staining from epithelium (arrows), although the cornified layer retains high background. Bars for panels A–H are equivalent. Bars=40 μm.

### TABLE 2. Effects of HMG-CoA Reductase Inhibitors on HMG-CoA Reductase Immunofluorescent Staining Intensity in Rat Esophageal Keratinocytes After 14 Days

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean IF intensity*</th>
</tr>
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<tbody>
<tr>
<td>Methyl cellulose</td>
<td>3.0</td>
</tr>
<tr>
<td>MK-803 (lactone of lovastatin)</td>
<td>3.0</td>
</tr>
<tr>
<td>L-154,819 (lovastatin hydroxyacid)</td>
<td>3.0</td>
</tr>
<tr>
<td>MK-733 (lactone of simvastatin)</td>
<td>2.4</td>
</tr>
</tbody>
</table>

*Mean values of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase immunofluorescence (IF) intensity were determined on frozen sections of rat esophageal keratinocytes as described in the footnote to Table 1; five rats were measured per group.

### TABLE 3. Reversibility of L-154,819-Induced HMG-CoA Reductase Immunofluorescent Staining Intensity in Rat Forestomach Keratinocytes

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean IF intensity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-154,819, 30 mg/kg/day</td>
<td>4.0</td>
</tr>
<tr>
<td>L-154,819, 75 mg/kg/day</td>
<td>5.0</td>
</tr>
<tr>
<td>L-154,819, 30 mg/kg/day, 14-day recovery</td>
<td>0.6</td>
</tr>
<tr>
<td>L-154,819, 75 mg/kg/day, 14-day recovery</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Mean values of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase immunofluorescence (IF) intensity were determined on frozen sections of rat forestomach keratinocytes as described in the footnote to Table 1. All rats were treated with the indicated HMG-CoA reductase antagonists for 7 days; three animals were sampled per group.
Figure 2. Transmission electron photomicrographs of forestomach keratinocytes from control rats given 0.5% methylcellulose for 14 days. Panel A: Low-magnification photomicrograph of forestomach epidermal epithelium, depicting normal stratum corneum (sc) and subadjacent stratum granulosum (sg). Corneocytes of the sc have few organelles and display prominent dense intercellular material (arrow). Keratinocytes of the sg contain numerous lamellar bodies (arrowheads) at their apexes but exhibit little interstitial material at their lateral and basal surfaces. Panel B: Higher-magnification view of stratum corneum intercellular space shows the layered configuration (arrowhead) of its lipid material. Panel C: Forestomach lamellar bodies are composed of stacks of lipid disks (arrowheads) and an outer bilaminar membrane (arrow). Bar for panel A = 1 μm, and for panels B and C, 0.1 μm.
FIGURE 3. Transmission electron photomicrographs of forestomach keratinocytes from rats treated with lovastatin hydroxyacid (L-154,819) at 75 mg/kg/day for 14 days. Panel A: Low-magnification view of stratum corneum (sc) and stratum granulosum (sg). Many conspicuous gaps (arrowheads) are present in interstitial lipid layers of the sc, and sg keratinocytes show a striking reduction in their content of lamellar bodies. Panel B: Higher-magnification view of the sc/sg interface. Intercellular space exhibits gaps devoid of lipid lamellae (arrows), and clusters of heterogeneous vesicles (v) are present instead. Most lamellar bodies of sg keratinocytes contain atypical vesicles of varying size (arrowheads) that lack lipid disks, but a few lamellar bodies containing internal lamellae do persist (l). Panel C: Photomicrograph similar to that in panel B, depicting lamellar bodies with residual lipid disks (arrowheads) and vesicles and heterogeneous spherules in intercellular space (arrows). Bar for panel A = 1.0 μm, and for panels B and C, 0.2 μm.
of HMG-CoA reductase IF staining in their hyperplastic forestomach keratinocytes. Administration of L-154,819 at 75 mg/kg/day for 14 days followed by 14 days of abstinence resulted in complete reversibility of the atypical effects of the hydroxylacid. A continuous zone of intercellular material composed of stacked lipid lamellae was present at the stratum corneum–stratum granulosum interface, and lamellar bodies of the stratum granulosum were normal in structure, size, and number (Figure 4A and Table 4). HMG-CoA reductase IF of these keratinocytes had also fallen to control values (Table 3). Rats treated with lovastatin lactone (MK-803, 75 mg/kg/day for 14 days) exhibited lamellar bodies and intercellular sheets of lipid indistinguishable from those observed in controls (Figures 4B and 4C and Table 4). Likewise, the HMG-CoA reductase IF of their forestomach keratinocytes was not elevated, and no forestomach keratinocyte hyperplasia was observed (Table 1).

Discussion

In these experiments rats treated orally with high doses of certain potent HMG-CoA reductase inhibitors accumulated elevated concentrations of HMG-CoA reductase IF in their forestomach keratinocytes. The occurrence of intense keratinocyte HMG-CoA reductase IF in controls (Figures 4B and 4C and Table 4) and histological alterations than its hydroxyacid, despite almost total conversion of the lactone to the open acid in rodent plasma.29 In addition, TEM of forestomach keratinocytes demonstrated dramatic alterations in the ultrastructure of lamellar bodies and stratum corneum intercellular lipid material after administration of lovastatin hydroxylacid at 75 mg/kg/day for 14 days. Lamellar bodies were less frequent, had lost most of their internal lipid lamellae, and exhibited numerous internal vesicles. Keratinocytes containing these atypical lamellae were situated in the stratum granulosum, the precise site of greatest HMG-CoA reductase IF induction in the forestomach epithelium of L-154,819–treated rats. Similarly, lipid disks were notably absent from the intercellular spaces of the stratum corneum, especially at its junction with the stratum granulosum. The latter effects were reversed completely after discontinuing administration of L-154,819. In contrast, lovastatin lactone (the prodrug) failed to produce such changes. These ultrastructural deviations did not occur after administration of a lower dose of L-154,819 (30 mg/kg/day) despite the induction of HMG-CoA reductase IF, suggesting that TEM changes in lamellar bodies are the end stage of a continuum of biochemical reactions resulting from prolonged inhibition of HMG-CoA reductase in rat forestomach keratinocytes.

It has long been accepted that the stratum corneum constitutes a highly impermeable barrier that affords protection against water loss. This property is probably due to lipids (secreted from lamellar bodies) that fill its intercellular spaces.21,30 Furthermore, the epidermis is
a major site of steroidogenesis, and 15–30% of the mass of epidermal lamellar bodies is composed of sterols or their derivatives. As expected from these observations, disruption of the murine epidermal permeability barrier leads to increased keratinocyte sterol synthesis during barrier recovery. In addition, certain epidermal alterations (neutral lipid storage disease and essential fatty acid deficiency) are characterized by keratinocyte hyperproliferation, increased levels of corneocyte desquamation, and breakdown of the epidermal permeability barrier. In both instances, keratinocyte lamellar bodies were abnormal and sheets of lipid were lacking from the intercellular spaces of the stratum corneum, just as was observed here in the forestomachs of rats treated with lovastatin hydroxyacid. Also, subsequent to the completion of our exper-
ments, we became aware of recent confirmatory studies regarding the effects of lovastatin on the hairless mouse epidermis. Topical application of lovastatin to the flank inhibited keratinocyte cholesterol synthesis after barrier disruption, prevented normal barrier recovery, induced epidermal hyperplasia, and disrupted lamellar body ultrastructure. Collectively, these studies and the results presented here support the hypothesis that normal rodent keratinocyte structure, function, and turnover depend on adequate regulation of sterol synthesis.

Recently, additional biochemical studies have demonstrated that the mouse forestomach epidermis is rich in 7-dehydrocholesterol, which disappeared during development of hyperkeratosis/acanthosis after administration of L-154,819. Increased cholesterol synthesis and HMG-CoA reductase activity were also detected after administration of L-154,819, thus confirming our IF data. Together with our findings, these results strongly suggest that the status of keratinocyte sterol synthesis affects the structure and function of lamellar bodies and that secretion of atypical lipids into the stratum corneum markedly enhances corneocyte desquamation, thereby stimulating epidermal hyperkeratosis/acanthosis. Maintenance of high local concentrations of lovastatin hydroxyacid or simvastatin in the rat forestomach via oral administration probably inhibits newly synthesized HMG-CoA reductase. As a consequence, epidermal sterol (e.g., 7-dehydrocholesterol) synthesis falls, resulting in the secretion of atypical proportions of lipids and sterols into the intercellular spaces of the forestomach epidermis, which probably causes hyperkeratosis. The occurrence of rodent forestomach alterations in response to lovastatin hydroxyacid and simvastatin thus appears to be dependent on the mechanism of action of these potent HMG-CoA reductase inhibitors.

References

27. Kloss MW, Patrick DH, MacDonald JS: The effects of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors on the rodent forestomach (abstract). Toxicologist 1990;10

KEY WORDS • lovastatin • 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors • keratinocyte hyperproliferation • electron microscopy
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