Histologic Changes in the Skin of Hairless Mice Following Peeling With Salicylic Acid

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Objective: To clarify the histologic alterations produced by the application of salicylic acid solution, which has been used effectively in chemical peeling without producing a wound or inflammation.

Design: We applied 7.5%, 15.0%, and 30.0% salicylic acid in solutions of ethanol or macrogol to the backs of hairless mice for 20 minutes. The skin was histologically evaluated immediately and at 1, 3, 12, 24, and 48 hours following treatment.

Setting: The Department of Dermatology, Faculty of Medicine, Kyushu University, Fukuoka, Japan.

Main Outcome Measures: A loss of cornified cells was the only morphologic alteration associated with the treatment, and was followed by the activation of the epidermal basal cells and the underlying fibroblasts.

Results: The 7.5% and 15.0% salicylic acid solutions produced few histologic changes, whereas the 30.0% salicylic acid in both vehicles macerated and then exfoliated the cornified cells. As the epidermis became thinner, the residual epidermal cells became flattened and were rearranged parallel to the tensile surface load. The cornified material within the hair follicles also became macerated, dilated the follicles, and then dropped off. An apparent increase occurred in the number of cells in the S phase in the epidermal basal cells in 24 hours, leaving the follicular cells unchanged. As the cornified layer thickened in 48 hours, the epidermal cells below it and the underlying fibroblasts resumed their random pretherapy arrangement. Except for the occasional infiltrate of lymphocytes, no degenerative or inflammatory changes occurred. While similar changes occurred with each vehicle, they were relatively faster with the ethanol preparations.

Conclusion: The present results suggest that the architecture of the epidermis and the papillary dermis can be regenerated by simply injuring the cornified layer by using topical agents such as salicylic acid that do not cause degeneration or inflammation.

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Chemical peeling has been used effectively to obtain the so-called rejuvenation of the skin, achieving a renewal of the epidermis with a rebuilding of the dermal connective tissue. Variously used are phenol, trichloroacetic acid, α-hydroxy acids, and salicylic acid. These acidic solutions chemically denature, and ultimately lead to the peeling away of parts of the epidermis and the dermis. Although controlled wounding and inflammation are thought to lead to regeneration of the epidermis and the connective tissue during healing, the mechanisms responsible for the cosmetic effects of chemical peeling have not been satisfactorily explained. Clinical and experimental studies suggest that degeneration or inflammation is not required to achieve the epidermal as well as the dermal regeneration. The connective tissue is produced mainly by the fibroblasts. Its construction is modulated by various molecules, including glycosaminoglycans, growth factors, and cytokines secreted from the overlying epithelium. Thus, certain changes in the epidermis produced by the application of chemicals could alter the underlying connective tissue without directly wounding the tissue or causing inflammation. If this were the case, one could safely induce chemical peeling of the skin of Asians, who are at a higher risk for pigmentary disorders and keloids than most whites. Wounding is associated with a risk of scarring.

We conducted this study to evaluate the changes in the skin 48 hours after the application of salicylic acid solutions. It has been reported that the application of salicylic acid in ethanol produces epidermal hyperplasia in 2 days, and suggested that
MATERIALS AND METHODS

We used 12 male albino, hairless mice, aged 8 weeks. Following established protocol, 12 3 concentrations of salicylic acid (7.5%, 15.0%, and 30.0%) were dissolved in 99% ethanol (pH 2.04) or in macrogol (pH 1.22).

Each step of the experiment was performed in animals that had been anesthetized by the intraperitoneal injection of pentobarbital. After cleansing with 70% ethanol, the back of each animal was marked with a pen to divide it into 6 squares (12 × 12 mm) with a 3-mm margin. To the 3 squares on the right side we applied 7.5%, 15.0%, and 30.0% salicylic acid, respectively, in ethanol. To the 3 squares on the left side we applied 7.5%, 15.0% and 30.0% salicylic acid, respectively, in macrogol. As a control, only the ethanol or macrogol vehicle was applied. After 20 minutes, the solutions were rinsed away with distilled water and the skin was gently dried with cotton swabs. No changes were grossly apparent on the treated skin at that time.

TISSUE PREPARATION

Under anesthesia, we excised the 6 treated squares from the backs of the animals, which were then killed by the further injection of pentobarbital. The specimens were obtained before and immediately following treatment and at 1, 3, 12, 24, and 48 hours. Each specimen was cut in half. One half was snap-frozen in liquid nitrogen, embedded in ornithine carbamyl transferase (OCT) compound, cut to 4-µm thickness, and stained with hematoxylin-eosin. These frozen sections were used to evaluate the morphometry of the cornified cell layer, since freezing preserves the skin structures that are sensitive to dehydration.13

The other half of the specimen was fixed with 10% formaldehyde in phosphate-buffered solution (PBS) (pH 7.2), embedded in paraffin, cut to 4-µm thickness, and stained with hematoxylin-eosin. These sections were used to evaluate the histologic characteristics of the skin according to the standards established for paraffin-embedded sections. In addition, immunohistochemical analysis was performed to detect proliferating cells using an antibody against proliferating cell nuclear antigen (PCNA) (Oncogene Science Inc, Uniondale, NY). In brief, paraffin sections were incubated with a 1 × 10^{-3} dilution of peroxidase-conjugated anti-PCNA antibody after blocking nonspecific reactions. Specimens were further incubated with diaminobenzidine tetrahydrochloride (Nacalai Tesque, Kyoto, Japan) and hydrogen peroxide in the same buffer solution for 3 minutes.

EVALUATION OF TISSUE SPECIMENS

We measured the thickness of the cornified cell layer and that of the lower layer of the epidermis from the granular to the basal layer using the frozen sections. Paraffin-embedded sections were used to identify the presence of degenerative and/or inflammatory changes and to evaluate the histopathologic features of the treated skin. The PCNA-stained sections were used in counting the cells in the late S phase.14 The thickness of each layer and the number of PCNA-positive cells (per 10 basal cells) in the epidermis were determined by measuring 10 different points under a light microscope (BH-2; Olympus, Tokyo, Japan) that was equipped with a scaled ocular lens.

Data are reported as mean±SD. We used SAS statistical software (SAS Institute Inc, Cary, NC). The extent of edematous change, the number of infiltrated cells, and the presence of immunoreactive cells in the connective tissue were scored as absent (−), occasionally present (+/−), or usually present (+).

RESULTS

TREATMENT WITH 7.5% AND 15.0% SALICYLIC ACID

The frozen and formaldehyde-fixed specimens of skin showed few histologic differences in the areas treated with 7.5% or 15.0% salicylic acid dissolved in ethanol or macrogol compared with the control areas at 48 hours' follow-up.

TREATMENT WITH 30.0% SALICYLIC ACID

Immediately after treatment with 30.0% salicylic acid, the cornified cell layer showed a temporary thickening up to 2.5 times the pretreatment thickness (Table; Figure 1A-B). The cornified layer then thinned markedly within 1 hour, and became further attenuated up to 12 hours after treatment, showing a thickness one half of that before treatment (Table; Figure 1C). The attenuation of this cornified layer was mainly caused by a shedding of the shrunken cornified cells from the skin surface. Interestingly, the thinning was accompanied by a rearrangement of the residual epidermal cells below the cornified layer into a more regular configuration, particularly the basal cells (Figure 1C), compared with the random arrangement prior to treatment (Figure 1A). At 48 hours after treatment, the epidermis began to thicken, and had accumulated several layers of cornified cells (Table; Figure 1D). At this time, the epidermal cells below that layer exhibited the same random arrangement as existed before therapy.

Cornified cells plugged the hair follicles before treatment (Figure 1A and Figure 2A). These plugs also became macerated, and gradually dilated the pores of the individual hair follicles (Figure 1B-D). In the formaldehyde-fixed sections, the macerated plugs became detached over the 48 hours leaving the granular cells intact (Figure 2B-D).

Interestingly, the frozen and formaldehyde-fixed sections exhibited unique artifacts, respectively. The frozen sections, following each of the ethanol and the macro-
Histologic Findings in the Skin of Hairless Mice Treated With 30.0% Salicylic Acid in Ethanol or Macrogol Solution*

<table>
<thead>
<tr>
<th>Time of Evaluation</th>
<th>Thickness of Cornified Layer, µm</th>
<th>Thickness of Granular to Basal Layer, µm</th>
<th>No. of PCNA-Positive Cells per 10 Basal Cells</th>
<th>Edema</th>
<th>Infiltrates</th>
<th>PCNA-Positive Cells</th>
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</thead>
<tbody>
<tr>
<td>Before Treatment</td>
<td>10.8 ± 3.8</td>
<td>30.0 ± 3.9</td>
<td>2.7 ± 1.7</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
</tr>
<tr>
<td>After treatment, h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>E</td>
<td>M</td>
<td>+/−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
</tr>
<tr>
<td>1</td>
<td>11.6 ± 3.9</td>
<td>13.6 ± 4.3</td>
<td>28.4 ± 4.0</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
</tr>
<tr>
<td>3</td>
<td>7.6 ± 2.3</td>
<td>5.6 ± 2.1</td>
<td>25.6 ± 6.0</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>12</td>
<td>6.4 ± 2.1</td>
<td>5.2 ± 1.9</td>
<td>16.4 ± 3.0</td>
<td>+/−</td>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
<td>24</td>
<td>8.4 ± 2.3</td>
<td>10.4 ± 4.7</td>
<td>21.2 ± 3.8</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
<td>48</td>
<td>10.4 ± 2.7</td>
<td>12.5 ± 4.7</td>
<td>27.7 ± 5.3</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
</tr>
</tbody>
</table>

*PCNA indicates proliferating cell nuclear antigen; ellipses, not applicable; E, ethanol solution; M, macrogol solution; +, usually present; −, absent; and +/− occasionally present.

Figure 1. Micrographs of frozen specimens obtained before (A) and immediately after (B) the application of the 30.0% salicylic acid in ethanol solution, 12 hours after application of the salicylic acid in ethanol solution (C), and 24 hours after application of the salicylic acid in macrogol solution (D). A. Before treatment, the surface of the epidermis appears irregular. While the cornified layer is sensitive to dehydration, it has been well preserved in the frozen sections. B. Immediately after treatment, the epidermis exhibits a temporary thickening of the cornified cell layer. Flat vacuoles 20 to 30 µm wide are seen in the granular cell layer. The presence of these vacuoles apparently increased the thickening of the epidermis. C. Twelve hours after application of the salicylic acid in ethanol solution, the epidermis appears uniformly thin and lacks the shallow grooves on its surface that represent epidermal “glyphics.” D. Twenty-four hours after application of the salicylic acid in macrogol solution, the skin shows a thickening of the granular cell layer with occasional mitosis of the basal cells. These changes developed relatively slowly following application of the salicylic acid in macrogol solution.

gol vehicle treatments, developed flattened vacuoles 20 to 30 µm wide beneath the cornified layer (Figure 1B). These vacuoles occurred only in the sections obtained immediately after treatment, and no pathologic changes occurred suggestive of degeneration or inflammation (Figure 1C-D). In the formaldehyde-fixed specimens (Figure 2), the cornified cell layer seemed to become cleaved above the granular layer in the 12 to 48 hours following treatment (Figure 2C-D), where the vacuoles had been seen in frozen sections (Figure 1B). The clefts represented an impairment of the cohesion of the cornified and the granular cells, although such cleavage could have been artificially produced during the dehydration.

Results of immunohistochemical study revealed the presence of PCNA-positive cells in the basal layer of the epidermis at a ratio of 2.7 ± 1.7 cells per 10 basal cells in the control as well as in the treated skin (Table; Figure 3A). The number of PCNA-positive basal cells showed an increase 12 hours after treatment. At 24 hours after treatment, the number of PCNA-positive basal cells

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had more than doubled to 6.7 ± 1.5 cells per 10 basal cells compared with pretreatment values (Table). It was interesting that PCNA-positive cells were uniformly present along the basal cell layer (Figure 3B). There was little increase in their number in the basal cells in the hair follicles. Mitosis of the basal cells occurred occasionally in the areas treated with salicylic acid in the ethanol and macrogol solutions. At 48 hours, the spindle-shaped cells beneath the epidermis occasionally showed PCNA positivity.

Slight edematous changes were seen in the papillary dermis of the skin treated with the salicylic acid in ethanol solution 1 hour after treatment. These changes persisted for 3 hours, and disappeared by 12 hours (Figure 1 and Figure 2). Similar changes occurred in the skin treated with salicylic acid in either the ethanol or macrogol vehicles. However, with the ethanol solution, the changes occurred relatively rapidly and disappeared quickly, in a few hours (Table). Lymphocytic infiltrates appeared only occasionally around the hair follicles in the treated areas of skin, and no degenerative changes occurred with any treatment throughout the observation period (Figure 1 and Figure 2).

**COMMENT**

Topical applications of salicylic acid have been used to remove excessively cornified skin, including corns and calluses, and to treat such systemic forms of hyperkeratosis as ichthyosis. Salicylic acid, an organic solvent, removes the intercellular lipids that are covalently linked to the cornified envelope that surrounds the cornified cells. Organic acids extract integral proteins from the desmosomes, including desmogleins, and thus destroy the cohesion of the epidermal cells. In addition, salicylic acid may activate certain pathways involved in the normal hydrolytic shedding of cornified cells. Thus, salicylic acid could cause a detachment of the cornified cells on the surface of the skin and in the hair follicles.

Salicylic acid solutions are used in chemical peeling to obtain a regeneration of the epidermis and dermis. Kligman observed epidermal hyperplasia 2 days after the application of salicylic acid in ethanol solution. In mouse experiments, a similar procedure led the epidermal basal cells into the S phase and even into mitosis in 24 hours, and activated the underlying fibroblasts in 48 hours. However, salicylic acid exhibits an antihyperplastic effect on the epidermis, and shows little effect on the mitotic activity of the human epidermis. Thus, it has been used in treating the hyperkeratotic disorders.

The effectiveness of chemical peeling is related largely to the extent of the injury induced. However, injury to the superficial epidermis occurs with all types of chemical peeling. This is also the case with laser peeling, which removes tissue by heating the superficial layer of skin. In the present study, a loss of cornified cells was the only morphologic alteration associated
with salicylic acid peeling, followed by the activation of the epidermal basal cells and the underlying fibroblasts. These findings suggest that the cornified layer plays a key role in maintaining the epidermal architecture. The epidermis is in a dynamic equilibrium, with the cornified cells continually being shed from the surface and being replaced by cells from the basal layer. During this process, the basal cells differentiate to cornified cells, become transformed from cuboidal to flattened cells, and become regularly arranged in a hexagonal (honeycomb) pattern that balances the tensile load of the body surface.21,22 Therefore, the epidermal cells below the cornified layer are relatively free from an effect of the various loads on the body surface (Figure 4A).23

Following damage to the cornified layer, the residual epidermal cells become subject to the tensile surface load that is normally shared by the layer (Figure 4B). Lacking a protective layer, the cells would lose water from their surface, thus increasing the tension. The destruction of cellular cohesion could cause an easy sliding and a deformation of the individual epidermal cells according to the load.18 These would explain the flattening and rearrangement of the epidermal cells parallel to the surface following treatment with salicylic acid (Figure 4C). Physical stress should be distributed evenly on the basal cells parallel to the surface, thus producing a uniform activation. Although mechanical receptors on the keratinocytes have not yet been determined,27 such a physical mechanism may explain the alterations in the absence of degeneration or inflammation. It has been reported that epidermopoiisis is related to the thickness of the epidermal surface being macerated.25

The production of such connective tissue components as collagen is regulated by factors secreted by the connective tissue cells as well as by cells of the overlying epithelium.9,10 The epithelium also modulates the 3-dimensional construction of collagen fibers by arranging glucosaminoglycans along the basement membrane.8 It is reasonable to infer that the dynamic and proliferative changes in the overlying epidermis stimulate the underlying fibroblasts. This would lend credence to previous reports that a remodeling of the superficial skin following chemical peeling may be unrelated to any degenerative or inflammatory changes.6,7,11 This could explain why the aged skin shows a marked benefit from chemical peeling. In aged skin, the cornified cells tend to remain adherent, thus producing an atrophic epidermis that is covered by a thin layer of cornified cells.26 Removal of the cornified layer would markedly deform the residual epidermis, which would facilitate its regeneration. On the
other hand, damage to the cornified layer of the skin of young people would cause minimal change since their cells are actively being shed.

The findings of this study suggest that impairing the cornified layer may have essential effect on the remodeling of the epidermis as well as of the papillary dermis. Inflammatory infiltrates occurred only occasionally under treatment with any preparations, but were relatively less frequent in the skin treated with salicylic acid in the macrogol vehicle. The burning pain sometimes encountered with the ethanol vehicle does not occur with macrogol. Therefore, the macrogol vehicle may be advantageous in chemical peeling, especially in Asian patients, who tend to develop hyperpigmentation or keloids.

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REFERENCES