Effect of Vitamin E on Keratinocyte-Modulation Induced by Lauroylsarcosine

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ABSTRACT—The effect of vitamin E on the modulation of keratinocytes was studied in rats. A 1% lauroylsarcosine (LS) ointment caused skin erythema with keratinocyte-damage. A 30% vitamin E ointment markedly alleviated this erythema and protected keratinocytes from cell damage. Vitamin E (100 µg/ml) was also effective on LS (7.5 µg/ml)-induced proliferative reduction of cultured keratinocytes. On the other hand, ointment containing superoxide dismutase (SOD) (99,000 U/g) decreased the LS-induced erythema, suggesting that superoxide anion (O$_2^-$) produced from keratinocytes play an important role in the skin irritation. Indeed, LS induced O$_2^-$ production from cultured keratinocytes. The O$_2^-$ was significantly reduced by vitamin E and SOD, although vitamin E had no effects on O$_2^-$ production in a xanthine-xanthine oxidase system, unlike the effect observed with SOD. These results indicate that vitamin E is an inhibitor of keratinocyte-modulation.

Keywords: Skin irritation, Superoxide anion, Vitamin E, Keratinocyte

Transdermal absorption enhancers are believed to play an important role for more extensive development of transdermal drug delivery systems including patches (1). However, they are often reported to have the disadvantage of causing skin irritation (2). Previously, we found a clue to overcome this problem, as vitamin E alleviated erythema induced by a transdermal absorption enhancer, lauroylsarcosine (LS), without blocking penetration of LS (3). In this paper, the effect of vitamin E on keratinocyte-modulation is studied to elucidate the mechanism whereby vitamin E decreases the erythema.

MATERIALS AND METHODS

Animals

Wistar male rats purchased from Japan SLC, Inc. (Hamamatsu) were subjected to investigation after housing for 7–10 days at constant temperature (24±2°C) and relative humidity (55±5%), under specific pathogen-free conditions with free access to food and water. These animals were sacrificed under ethyl ether anesthesia.

Reagents

LS (extra pure, Nacalai Tesque, Kyoto), vitamin E (alpha-tocopherol acetate) and superoxide dismutase (SOD) derived from bovine erythrocytes (3300 U/mg) (Wako Pure Chemicals, Osaka), and vinblastine sulfate salt (Sigma Chemicals, St. Louis, MO, USA) were used. The ointment containing compounds to be tested was prepared using hydrocarbon gel (Taisho Pharmaceutical Co., Tokyo) as a base. Thus, each compound was added to hydrocarbon gel at an appropriate dose, mixing in a mortar. The ointment was prepared just prior to use.

For the keratinocyte assay, vitamin E was dissolved in methyl alcohol to dilute it 1:50 with culture medium. Methyl alcohol at this concentration (0.2%) had no cytotoxicity.

Skin irritation testing

One-tenth gram of ointment containing LS, vitamin E or SOD was mounted on a round polymer film (a laminated film of polyethyleneterephtalate and ethylenevinyl acetate, 3.14 cm$^2$). It was applied onto the shaved back of a rat, weighing 150–180 g and fixed with gauze and bandage. After 24 hr, the ointment was removed and the score of erythema was determined according to the method of Draize (4) as follows: 1, mild erythema; 2, moderate erythema; 3, severe erythema. Vinblastine (0.75 mg/kg, i.v.) was administered 4 days before application of LS ointment. The number of polymorphonuclear leukocytes (PMN) in the tissue was determined by measuring the myeloperoxidase activity of the skin extract (5). The calcu-
lation of PMN in peripheral blood was performed microscopically using a hemocytometer. Specimens for microscopic observation were prepared as follows: Isolated skin was fixed with 10% neutral formalin solution, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin.

**Preparation of keratinocytes**

Keratinocytes were prepared from the shaved dorsal skin of rats weighing 30–50 g. The isolated skin was washed in phosphate-buffered saline at pH 7.2 (PBS) after cutting it into fragments of approximately 2 cm². The skin fragments were incubated in 0.25% trypsin dissolved in PBS for 18 hr at 4°C. Then they were further incubated for 2 hr at 37°C to peel off the epidermis. This epidermis sheet was stirred gently in PBS for 10 min at 37°C to separate the keratinocytes. The isolated keratinocytes were collected by centrifuging at 650 x g for 5 min and suspended in calcium-free Hank’s balanced solution (HBSS) to a concentration of 5 x 10⁶ cells/ml.

**Cytotoxicity test**

Packed keratinocytes were suspended in keratinocyte growth medium (KGM) (Clonetics, San Diego, CA, USA). A 1-ml aliquot of the keratinocyte suspension (1.5 x 10⁶ cells/ml) was inoculated into a well and cultured in an atmosphere of 5% CO₂ at 37°C. When the cells grew to 70% or 80% confluency, 2.5 ml of vitamin E was added to the well (100 μg/ml). After 2 hr, 2.5 ml of LS was added (7.5 μg/ml). The cells were cultured for 48 hr and then dispersed with 1% citrate in PBS to determine the number of cells.

**Measurement of O₂⁻ production**

The amount of O₂⁻ was determined by measuring the reduction of equine ferricytochrome C (6). In this assay, each compound was dissolved or suspended in HBSS. The cell suspension (400 μl) of keratinocytes was mixed with 20 mM CaCl₂ (50 μl), 10 mM ferricytochrome C (100 μl), vitamin E or SOD (50 μl) and LS (200 μl). The mixture was incubated at 37°C for 30 min. Then, the absorbance was monitored at 550 nm. The amount of O₂⁻ generated was calculated by the difference of absorbance from the reference using an absorbance coefficient of 22.6/mM per cm.

The O₂⁻ generation in a xanthine-xanthine oxidase system was assayed as follows (6): A 100-μl aliquot of ferricytochrome C (10 mM) was mixed with 5 mM xanthine (100 μl), 160 U/ml xanthine oxidase (200 μl) and vitamin E or SOD (600 μl). After incubation for 10 min at 37°C, the amount of O₂⁻ generated was measured as described above.

**Statistical analyses**

The values are means ± S.E. statistically analyzed by Student’s t-test or Dunnett’s multiple comparison test, and P values < 0.05 were regarded as significant.

**RESULTS**

**Effect on LS-induced skin irritation**

Vitamin E or SOD was applied onto rat shaved dorsal skin together with LS (Table 1). As described previously (3), vitamin E was markedly effective on skin irritation of score 2 produced by 1% LS, as erythema scarcely appeared in any of six animals. In addition, SOD, a scavenger of O₂⁻, also decreased the erythema at a concentration of 99,000 U/g; however, it was less effective than 30% vitamin E. This suggests that O₂⁻ is involved in the appearance of erythema.

The effect of vinblastine is shown in Table 2. The intravenous injection of 0.75 mg/kg vinblastine decreased the number of PMN in the peripheral blood of rats. It resulted in the significant decrease of infiltrated PMN at

**Table 1. Effects of vitamin E and SOD on LS-induced erythema**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Score 0</th>
<th>Score 1</th>
<th>Score 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% LS</td>
<td>0/6</td>
<td>0/6</td>
<td>6/6</td>
</tr>
<tr>
<td>1% LS + 30% Vitamin E</td>
<td>6/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>1% LS + 99,000 U/g SOD</td>
<td>0/6</td>
<td>4/6</td>
<td>2/6</td>
</tr>
</tbody>
</table>

Each figure indicates the result of six animals.

**Table 2. Effect of vinblastine on LS-induced erythema**

<table>
<thead>
<tr>
<th>Treatment (i.v.)</th>
<th>Number of PMN in peripheral blood (× 10⁵ cells/ml)</th>
<th>Number of infiltrated PMN at LS-applied site (× 10⁵ cells/site)</th>
<th>Score of erythema</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>35.9 ± 1.6</td>
<td>2.5 ± 0.4</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Vinblastine (0.75 mg/kg)</td>
<td>3.8 ± 0.5***</td>
<td>1.5 ± 0.1*</td>
<td>1.8 ± 0.2</td>
</tr>
</tbody>
</table>

Each figure indicates the mean ± S.E. of five animals. ***P < 0.001, *P < 0.05: significantly different relative to the saline treated cells by Student’s t-test.
the LS-applied site of the skin. However, the compound had no effects on LS-induced erythema.

**Effect of vitamin E on LS-induced microscopic changes**

A microscopic study was performed 24 hr after ointment application. As shown in Fig. 1, 1% LS ointment induced degenerative changes of surface epithelium including necrosis. A slight vasodilation and perivascular infiltrates in the dermis were also seen. However, these findings were scarcely observed at the site applied with the combined ointment of 1% LS and 30% vitamin E, suggesting that vitamin E might be a potent inhibitor of keratinocyte-modulation.

**Effect of vitamin E on LS-induced keratinocyte damage**

LS caused a dose-related cytotoxicity on cultured keratinocytes, indicating that 7.5 μg/ml LS is an appropriate dose for studying the protective effect of vitamin E. The proliferation of keratinocytes was reduced about 80% by the addition of LS (7.5 μg/ml). Vitamin E (100 μg/ml) decreased this proliferative reduction, recovering the proliferation to about 80% that of the control (Fig. 2).

**Effect of vitamin E on LS-induced O$_2^-$ production from cultured keratinocytes**

A dose-related production of O$_2^-$ from cultured keratinocytes was induced following LS exposure. The O$_2^-$ of 0.23 μmol/min produced by the addition of LS (200 μg/ml) was almost completely eliminated by SOD (33 U/ml), a scavenging enzyme. A similar effect was also seen with vitamin E; It showed a significant effect on the O$_2^-$ production with an inhibitory percentage of about 60% at the dose of 100 μg/ml (Table 3).
Table 3. Effect of vitamin E on LS-induced O2- production from cultured keratinocytes

<table>
<thead>
<tr>
<th>Compounds</th>
<th>O2- production (µmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS (µg/ml)</td>
<td>SOD (U/ml)</td>
</tr>
<tr>
<td>-----------------</td>
<td>------------</td>
</tr>
<tr>
<td>200</td>
<td>0.225 ± 0.030</td>
</tr>
<tr>
<td>200</td>
<td>0.008 ± 0.050*</td>
</tr>
<tr>
<td>200</td>
<td>0.187 ± 0.028</td>
</tr>
</tbody>
</table>

Each figure indicates the mean ± S.E. of five experiments. *P < 0.05: Significantly different relative to the LS control by Dunnett’s test.

Table 4. Effects of vitamin E and SOD on xanthine-xanthine oxidase O2- generation

<table>
<thead>
<tr>
<th>Compound</th>
<th>O2- generation (µmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.78 ± 0.02</td>
</tr>
<tr>
<td>Vitamin E, 100 µg/ml</td>
<td>1.28 ± 0.04</td>
</tr>
<tr>
<td>SOD, 33 U/ml</td>
<td>0.25 ± 0.06*</td>
</tr>
</tbody>
</table>

Each figure indicates the mean ± S.E. of five experiments. *P < 0.05: significantly different relative to the control by Dunnett’s test.

**Effects of vitamin E and SOD on xanthine-xanthine oxidase O2- generation**

The effect of vitamin E was compared with that of SOD in a xanthine-xanthine oxidase O2- generation system. O2- of 1.78 µmol/min was produced in the control. SOD at 33 U/ml significantly inhibited this O2- generation with a suppression of more than 80%. However, no significant inhibition was observed for vitamin E at 100 µg/ml (Table 4).

**DISCUSSION**

The experiments were performed to elucidate the possible role of vitamin E on LS-induced skin irritation. The results presented here demonstrate that vitamin E is an inhibitor of keratinocyte-modulation. As described in our previous report (3), 1% LS-induced irritation was microscopically characterized as follows: During the early phase of 1 to 3 hr after LS application, keratinocyte-damage was the predominant finding. This appeared more markedly with time, concomitantly with vasodilation. Then at 6 and 24 hr, infiltration of PMN was observed in the dermis just under the damage of keratinocytes. Moreover, it was demonstrated that the score of erythema developed with the potency of vasodilation. From these results, some experiments were performed to test the postulation that vitamin E might be active for the modulation of keratinocytes and PMN. We initially investigated the involvement of PMN in the appearance of erythema using vinblastine which decreased the number of infiltrated PMN by reducing the division of bone marrow cells (7). Although PMN is reported to play an important role in some types of contact dermatitis (8, 9), vinblastine had no effects on LS-induced erythema, suggesting that keratinocytes might be more essential than PMN for the appearance of erythema induced by LS. Indeed, vitamin E, a potent alleviator of the erythema, was observed to protect against the keratinocyte-damage during the period when there was no PMN infiltration. These observations suggest that vitamin E might modulate keratinocytes and alleviate the erythema. Evidence for this was provided by the data showing that vitamin E was effective on both cell damage and O2- production induced by LS, suggesting that vitamin E is an inhibitor of keratinocyte-modulation. The suppression of O2- production is an especially important finding because the O2- scavenger SOD decreased LS-induced erythema as reported previously (10), indicating that O2- is a major mediator responsible for the skin irritation, because some reports have demonstrated that O2- plays a predominant role in the progress of skin inflammations (11, 12). Hence, vitamin E is considered to alleviate the erythema by suppressing O2- production from keratinocytes at an early phase of LS-induced irritation. Although the more detailed actions of vitamin E are unclear, the catalyzing action of O2- generally accepted as an activity of vitamin E might scarcely attribute to the suppression of O2- production. This is because vitamin E was ineffective on O2- generation in a cell-free system of xanthine-xanthine oxidase unlike the effect observed with SOD. These findings suggest that a possible role of vitamin E may be an inhibitor of cell membrane-modulation. This is compatible with the report that vitamin E inhibits O2- production from activated macrophages, probably through an interaction with membrane lipids (13). In addition, Kamimura (14) suggested that vitamin E has an effect on mast cell membrane-modulation during development of skin irritations caused by applications of croton oil and plaster. However, some further experiments should be carried out to reveal the effect of vitamin E on keratinocyte membrane-modulation.

Finally, it would be expected that this study should be significant for the understanding of the pathogenesis and management of skin irritation induced by chemicals.

**REFERENCES**

Drug Dev Ind Pharm 13, 589–651 (1987)


4 Draize JH: Appraisal of the Safety of Chemical in Foods, Drugs and Cosmetics, by the Staff of the Division of Pharmacology Food and Drug Administration, Department of Health Education and Welfare, FDA-Officials of US Business Office, Topeka, Kansas, p 46 (1959)


14 Kamimura M: Antiinflammatory activity of vitamin E. J Vitaminol 18, 204–209 (1972)