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Research Papers

Effects of vitamin E and squalene on skin irritation of a transdermal absorption enhancer, lauroylsarcosine

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Summary

Alleviators of skin irritation were sought and their practical usefulness was evaluated in rats during the development of reaction to lauroylsarcosine (LS), an enhancer of transdermal absorption. LS (0.04–5%) produced erythema in a dose-related manner. Microscopic findings suggested that the damage by LS on keratinocytes caused this skin irritation. In a cultured fibroblast model, vitamin E and squalene were screened as reducers of LS-induced cell damage. Both compounds apparently alleviated 1% LS-induced erythema. Moreover, the combined addition of vitamin E or squalene and LS to isosorbide dinitrate (ISDN) ointment maintained greater penetration and less irritation.

Introduction

The transdermal patch is generally accepted as a new type of drug delivery system maintaining effective levels in blood (Frishman and Sharmann, 1986). However, this system suffers from a disadvantage, since drugs cannot easily penetrate through the skin into the blood stream. This lack of skin penetration results from a primary function of the skin which is to act as a barrier to prevent the body from invasion of chemical products and to maintain homeostasis. To overcome this problem, enhancers for transdermal absorption such as azone (Spruance et al., 1984; Sugibayashi et al., 1985), fatty acid (Cooper et al., 1985; Aungst et al., 1986) and surfuctants (Colo and Vitale, 1989; Kadir and Cohen, 1989) have been investigated. However, these compounds are known to induce a side effect of severe skin irritation (Quan et a., 1991). Alleviators for these skin irritations have been scarcely reported thus far. In this study, as an approach to reduce skin irritation, the effects of vitamin E and squalene have been investigated for the erythema caused by lauroylsarcosine of a surfactant enhancer (Aungst et al., 1986; Tsubota et al., 1987).

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Materials and Methods

Animals

Wistar male rats (6 weeks, 130–160 g) were purchased from Nihon SLC (Japan) and sub-

jected to investigation after housing for 7–10 days.

Compounds

Lauroylsarcosine (LS, extra pure) was purchased from Nacalai tesque (Japan). Vitamin E acetate (> 98% pure) was obtained from Wako Pure Chemical Industries, Ltd (Japan). Squalene (> 98% pure) and isosorbide dinitrate (ISDN, contains 60% lactose) were purchased from Sigma Chemical Co. (U.S.A.). ISDN was prepared by extraction with ethyl acetate, removing lactose by filtration and the solvent by evaporation.

Preparation of ointment

The ointment containing LS, vitamin E, squalene or ISDN was prepared using hydrocarbon gel (Taisho Pharmaceutical Co., Japan) as a base. Each compound was added to hydrocarbon gel at an appropriate dose, mixing in a mortar.

Cytotoxicity test

For this test, each compound was dissolved in methyl alcohol. Primary cultured fibroblasts were prepared from rat skin. The shaved rat skin was minced with scissors and washed with physiological buffered saline at pH 7.2 (PBS) to digest for 20 min at 37°C by 0.25% trypsin in PBS. The digestion was centrifuged at $670 \times g$ for 5 min after filtration. Packed cells were suspended in Eagle's Minimum Essential Medium containing 10% fetal calf serum (Flow laboratories, U.K.) and cultured in atmosphere of 5% CO_2. 1 ml of the tertiary cultured fibroblast suspension (1.5×10^5 cells/ml) was inoculated into a well and cultured for 16 h. Then, 2.5 μl of the compound to be tested was added to the well (10 μg/ml). 2 h later, 2.5 μl of LS was added (50 μg/ml). The cells were cultured for 48 h and dispersed with 1% citrate in PBS to determine the number of cells.

Skin irritation test

0.1 g of the ointment containing LS, vitamin E, squalene or ISDN was mounted on a round polymer film (3.14 cm^2, polyethylene-terephthalate-ethylenevinyl acetate). It was applied onto the shaved dorsal skin of a rat and fixed with gauze and bandage. After appropriate time intervals, the ointment was removed and the score of erythema was determined according to the method of Draize (1958) as follows: 1, mild erythema; 2, moderate erythema; 3, severe erythema. After macroscopic observation for erythema, the animals were killed and their dorsal skins removed. The sections from ointment-applied sites were fixed in 10% neutral buffered formalin for 7 days, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin-eosin for histopathologic examination.

Skin penetration test

The transdermal penetration of LS was studied by an in vitro test. The shaved dorsal skin was removed from a rat and placed in a Franz diffusion cell with application area of 3.14 cm^2 and receptor chamber of 15 ml volume (Franz, 1975). After filling the receptor chamber with PBS, 0.5 g of the ointment to be tested was applied to the skin. After 6 and 24 h, PBS in receptor chamber was taken to measure the amount of LS penetrated by high-performance liquid chromatography using with a column filled with Nucleosil 5C18 (Nippon Kuromatokogyo, Japan). The column was eluted with acetonitrile/50 mM KH_2PO_4 pH 2.0 (6:4, v/v) at a flow rate of 1 ml/min. The concentration of LS was determined by UV absorbance at 210 nm.

Plasma level of ISDN

The plasma was prepared from a rat exposed to ISDN ointment using heparin as an anticoagulant. This plasma (0.2 ml) was mixed with 3% bovine serum albumin in PBS (0.8 ml) and dichloromethane (5 ml). The mixture was centrifuged at $2500 \times g$ for 5 min after shaking for 12 min. After the upper layer was almost removed, the lower layer was completely separated from residual dichloromethane by freezing and dried under a nitrogen air flow. The dried preparation was dissolved in 10 μl of ethyl acetate to quantify ISDN content by gas chromatography on a Capillary column Ultra-1 (Hewlett Packard, U.S.A.). The flow rate of carrier gas (N_2) was 30 ml/min. The column temperature was maintained at 125°C during analysis. ISDN was detected using an electron capture detector (Shimadzu, Japan).

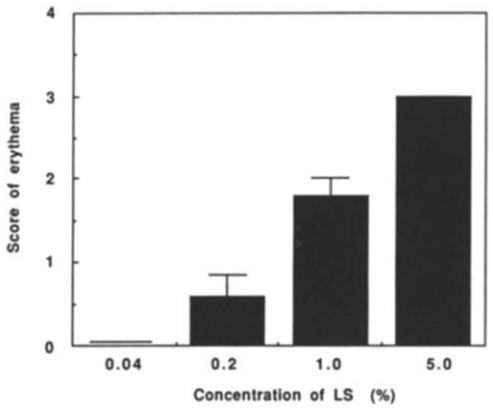

Fig. 1. Dose-relationship of LS-induced skin irritation in rats. The erythema was determined 24 h after application. Each column and vertical bar indicate the mean and S.E. of five animals.

Results

Microscopic characterization of LS-induced skin irritation

The ointment containing LS was applied onto the shaved backs of rats to estimate the irritative. LS (0.04–5.0%) produced an overt erythema in a dose-dependent manner (Fig. 1). Table 1 shows the macroscopic and the microscopic time course changes of 1% LS-induced irritation. At the early phase of 1–3 h after application of LS ointment, keratinocyte damage was a predominant finding. This became more marked with time, concomitantly with vasodilation. And then, at 6 and 24 h, infiltration with polymorphonuclear leukocytes (PMN) was observed in the dermis just under the damage caused by keratinocytes (Fig. 2). Moreover, it was demonstrated that the score of erythema developed with the potency of vasodilation (Table 1). These findings suggest that keratinocyte damage is a trigger of LS-induced erythema.

Effect on cell damage and skin irritation induced by LS

The cytotoxic effect of LS was examined for cultured fibroblasts derived from rat skin. LS produced dose-related cell damage, showing that a concentration of 50 μg/ml is the best to search for alleviators. With respect to LS-induced cell

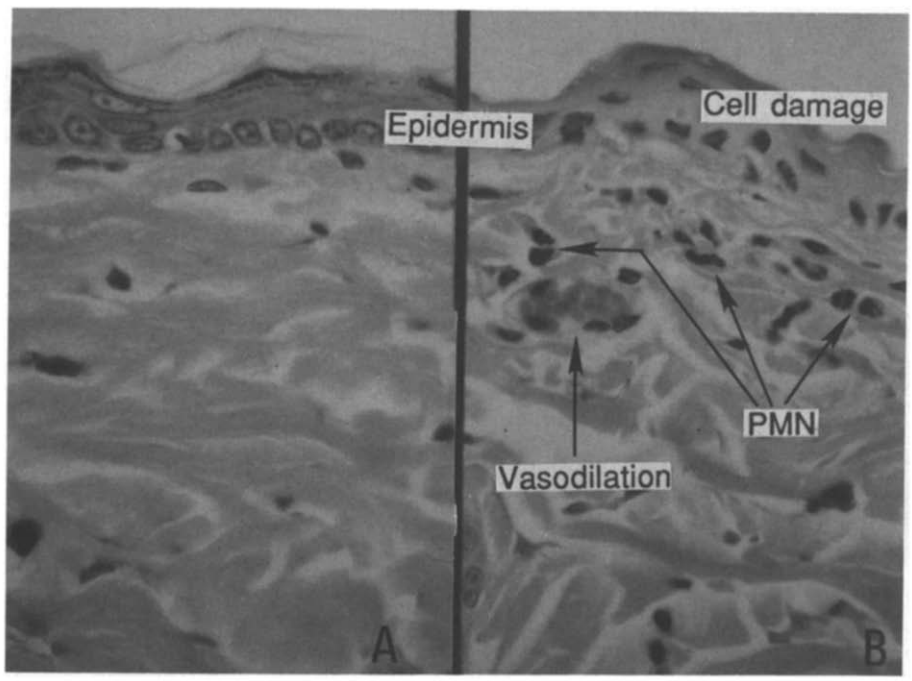

Fig. 2. Microscopic findings at 24 h after application of 1% LS ointment. A, normal skin; B, 1% LS-applied site.

TABLE 1

Macroscopic and microscopic time course changes of LS-induced skin irritation in rats

Time (h)	Damage of keratinocyte	Vaso-dilation	Infiltration with PMN	Score of erythema
1	+	−	−	0.2 ± 0.2
3	+ +	+	−	1.2 ± 0.2
6	+ + +	+	+	1.3 ± 0.2
24	+ + +	+ +	+ +	1.7 ± 0.2

Skin irritation was determined 1, 3, 6 or 24 h after application of 1% LS ointment. + + +, severe; + +, moderate; +, slight; −, not detectable. The score of erythema indicates the mean ± S.E. of six animals.

damage, vitamin E and squalene showed marked inhibitory effects at a concentration of 10 μg/ml (Fig. 3).

In a following experiment, the alleviation effect against skin irritation was studied for vitamin E and squalene in rats. The application of ointment containing 1% LS caused the erythema at a mean score of 2.4. The addition of squalene (2, 10%) induced an apparent reduction of the score. Vitamin E (10, 30%) showed more significant effects as erythema scarcely appeared at a concentration of 30% (Fig. 4).

Effect on transdermal penetration

The action of vitamin E and squalene on transdermal penetration of LS was examined in an isolated skin model. The amount of penetration from 1% LS ointment was about 1660 μg over 24 h. The addition of vitamin E (30%) or squalene (10%) to the ointment did not suppress this transdermal penetration of LS but instead enhanced it (Fig. 5). The flux value (μg/cm² h) was 22.0 ± 1.9 in 1% LS ointment, 34.5 ± 2.9 in 1% LS with 30% vitamin E and 52.0 ± 3.9 in 1% LS with 10% squalene, respectively.

Fig. 6 shows the practical usefulness of vitamin E and squalene in rats which were treated 6% with ISDN ointment. The blood level of ISDN was measured to estimate the amount of the

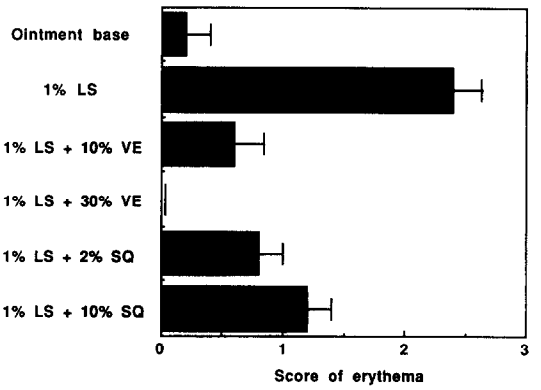

Fig. 4. Effects of vitamin E and squalene on LS-induced erythema in rats. The erythema was determined 24 h after application. Each column and vertical bar indicate the mean and S.E. of six animals.

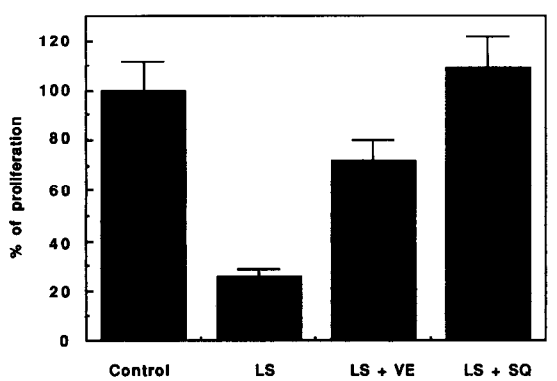

Fig. 3. Effects of vitamin E and squalene on LS-induced cell damage. Each column and vertical bar indicate the mean and S.E. of five experiments. LS, lauroylsarcosine (50 μg/ml); VE, vitamin E (10 μg/ml); SQ, squalene (10 μg/ml).

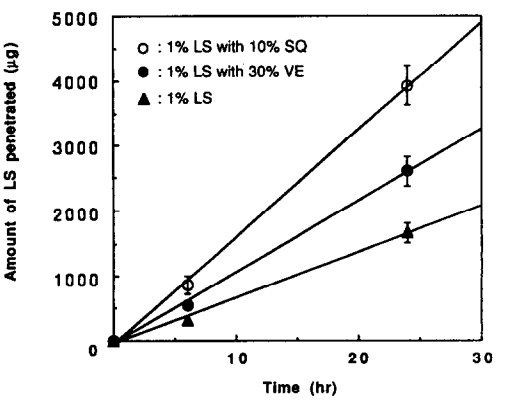

Fig. 5. Effects of vitamin E and squalene on transdermal penetration of LS. The amount of LS penetrated was measured 6 and 24 h after application. Each symbol and vertical bar indicate the mean and S.E. of four experiments.

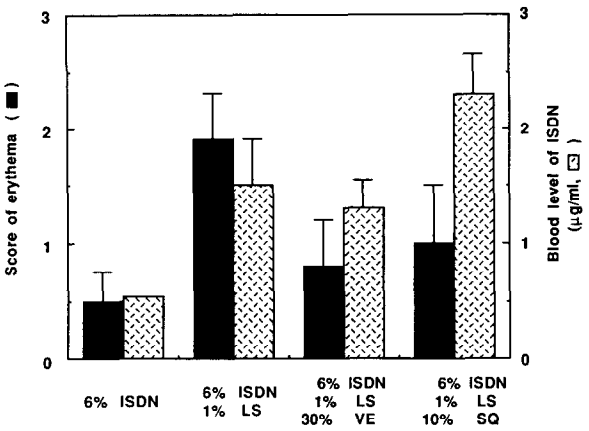

Fig. 6. Effects of vitamin E and squalene on the enhancement of ISDN penetration and skin irritation induced in rats by LS. The erythema or the blood level of ISDN was determined 24 h after application. Each column and vertical bar indicate the mean and S.E. of five animals.

compound which had penetrated through the skin while evaluating the irritative. The addition of LS to ISDN ointment raised blood levels about 3-fold and produced moderate to severe erythema. The combined addition of LS and vitamin E or squalene maintained or enhanced the effect of LS on the transdermal penetration of ISDN while alleviating the erythema.

Discussion

We searched for alleviators of skin irritation induced by LS, a transdermal absorption enhancer, and revealed that vitamin E and squalene apparently inhibited skin irritation while maintaining an enhancing effect on transdermal penetration. Although LS was firstly demonstrated in this laboratory to be an enhancer for transdermal penetration (Tsubota et al., 1987), as demonstrated here, it produces a skin irritation macroscopically characterized by erythema. In this paper, we searched for alleviators, taking the erythema as a principal indicator of skin irritation. From microscopic time course changes, we postulated that LS-induced keratinocyte damage initiated the skin irritation of erythema and that inhibitors of cell damage might alleviate the irritation. Then, considering that the action of LS on proteins or the lipid layer in the cell membrane may be responsible for the cytotoxicity, anti-inflammatory membrane stabilizers such as dexamethazone, indomethacin (Brown et al., 1967) and DSCG (Kuriyama and Okuno, 1981) and lipophilic compounds such as vitamins, triglyceride and cholesterol were subjected to an in vitro screening test of cultured fibroblasts. Of these compounds, both vitamin E and squalene were demonstrated to be potent inhibitors of LS-induced cytotoxicity and also to show overt alleviation on the erythema. Moreover, the addition of vitamin E or squalene to LS ointment containing ISDN was shown to alleviate LS-induced erythema without inhibiting the transdermal penetration of ISDN, suggesting an application to transdermal therapeutic systems. It is an especially significant revelation that vitamin E applied to many external drugs with established safety alleviated the skin irritation, although at high doses of 10–30%. These findings further suggest that the combination of a transdermal enhancer and an irritant alleviator would expand the range of drugs to be transdermally applied and help the development of a transdermal medicament maintaining greater penetration and less irritation.

The mode of action of vitamin E or squalene is as yet unclear, but as described above, their inhibitory activities on cell damage are postulated to be attributable to the alleviation of erythema. This postulation is supported by the evidence that vitamin E and squalene did not suppress the penetration of LS through the isolated rat skin and the result in a preliminary experiment that the damage of cultured keratinocytes induced by LS was inhibited by vitamin E and squalene. In addition, we speculate that LS-induced erythema might be caused by mediators generated from keratinocytes and that squalene or vitamin E might inhibit the generation or the release of mediators. Superoxide anion (O_2^-) is one of the possible mediators, since O_2^- has been reported to be a mediator of skin erythema in guinea pigs (Yoshioka et al., 1987). Further experiments along this train of thought are in progress in our laboratory.

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