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Research Paper

The Mode of Promoting Activity of *O*-Ethylmenthol as a Transdermal Absorption Enhancer

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Purpose. The mode of action of *O*-ethylmenthol (MET), a promising compound to enhance transdermal drug delivery, was elucidated. Morphology of the skin treated with MET was investigated employing a laser scanning confocal microscopy.

Methods. Confocal scanning laser microscope and laser scanning microscope were employed for the morphological evaluation of the stratum corneum. To evaluate the fluidity of intercellular lipids by treatment with MET, liposomes composed of the stratum corneum lipids were prepared.

Results. Distribution amounts of the fluorescent probes greatly increased in the intercellular regions of the stratum corneum treated with 40% ethanol containing MET. Based on the skin surface observations, the difference in relative height between keratinocytes and intercellular regions was defined as $\Delta H = \Delta H_{\text{keratinocytes}} - \Delta H_{\text{intercellular space}}$, where ΔH is the difference in relative height, $\Delta H_{\text{keratinocytes}}$ is the height of center region in the keratinocytes, and $\Delta H_{\text{intercellular space}}$ is the height of the intercellular space. ΔH values became negative in the skin surface treated with 40% ethanol containing MET because of the swelling in the intercellular regions. ΔH values changed from positive to negative 15–30 min after the administration of MET. A very short period of application of MET was sufficient to induce its promoting activity.

Conclusions. MET was able to change the structure of the intercellular lipids, thereby enhancing both the partitioning and diffusion of drugs through the skin.

KEY WORDS: confocal laser scanning microscopy; *O*-ethylmenthol; skin permeation; transdermal drug delivery.

INTRODUCTION

Utilization of a chemical enhancer is considered to be the most promising strategy to promote absorption rate through the skin. There are many reports that have investigated the effect of chemical enhancers (1,2). Furthermore, the promoting mechanism of chemical enhancers has been elucidated in numerous papers. It was found that cyclic monoterpenes such as *d*-limonene and *l*-menthol remarkably enhanced the skin permeation of drugs with the coexistence of ethanol. In our previous study, *O*-alkyl and *O*-acylmenthol (MET) derivatives were synthesized to increase the promoting activity, and we evaluated their promoting activity in the permeation of drugs through the skin. Among the synthesized compounds, *O*-ethylmenthol showed the greatest promoting activity and caused relatively little skin irritation (3). Using ketoprofen as a model drug, the promoting activity was compared with *l*-menthol in *in vitro* skin permeation study (4). The flux of ketoprofen was markedly increased by the administration of a small amount of MET. Furthermore, the partition parameter of ketoprofen obtained from the curve-fitting of permeation profile was increased. It means that MET increases the retention of drugs in the skin surface. Pretreatment of the skin with cyclic monoterpenes, such as *d*-limonene and *l*-menthol, increased the permeability coefficient of diclofenac (5). Moreover, these results suggested that the thermodynamic activity of absorption enhancers in the donor solutions were important to exhibit promoting activities. Recently, menthol derivatives, including MET, were evaluated for their structure–activity relationships employing an artificial neural network (6). Using one of the effective derivatives, 1-*O*-ethyl-3-butylcyclohexanol, the promoting mechanism was investigated (7). When the compound was applied to the reversed skin or stripped skin, almost no changes were observed compared to control. This result suggested that the compound's site of action was the stratum corneum. It was also clarified that the compound acted on the relatively hydrophobic region of the skin.

In this study, the mode of action of MET was elucidated in detail using confocal scanning laser microscopy, confocal scanning microscopy, lipid extraction, and stratum corneum lipid liposomes (SCLL).

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MATERIALS AND METHODS

Materials

MET was synthesized according to the method described by Meerwein *et al.* (8), and was characterized by elemental analysis, nuclear magnetic resonance (NMR) spectroscopy, and gas chromatography (GC) (Shimadzu GC-7A; Shimadzu Corp., Kyoto, Japan). The purity of this compound exceeded 99%. Fluorescein was purchased from Tokyo Chemical Industry, Co., Ltd. (Tokyo, Japan). Cholesterol CII test Wako, trypsin, and Mildform® 20N were purchased from Wako Pure Chemical Industries (Osaka, Japan). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI) was purchased from Lambda Probes & Diagnostics (Graz, Austria). Ceramide (type III from bovine brain sphingomyelin), cholesterol, palmitic acid, cholesterol-3-sulfate, and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma (St. Louis, MO, USA). Other chemicals used were of reagent grade.

Preparation of Donor Solution

Fluorescein (50 μM) was dissolved in phosphate-buffered saline (PBS) containing 40% ethanol and 0.5% MET. The pH of the solutions was adjusted to 7.4. DiI (10 μM) dissolved in dimethyl sulfoxide (0.1% of total volume of the solution) was dissolved in PBS containing 40% ethanol and 0.5% MET.

In Vitro Skin Distribution Study

The procedure was the same as in a regular permeation study. Briefly, full-thickness back skin was excised from male Hr-/Kud hairless mice (8 weeks old) and mounted in the two-chamber diffusion cell (available diffusion area, 0.785 cm^2; volume of each cells, 3.0 mL). The donor cell was filled with the solution containing the fluorescent probe and MET, and the receiver cell was filled with PBS. Fluorescein was used as a hydrophilic fluorescent probe and DiI was used as a lipophilic probe.

Observation Using Laser Scanning Confocal Microscopy (LSCM)

The skin was fixed with 10% formaldehyde 8 h after the application of donor solution containing the fluorescent probes. Next, the surface of the skin was observed using the LSCM (Radiance 2000, Bio-Rad Laboratories, Hercules, CA, USA).

Skin Surface Morphology

An adequate concentration of MET was administered to hairless mouse dorsal skin using a two-chamber diffusion cell. After an appropriate interval, the skin was removed from the cell. The skin cross sections were monitored with a charge-coupled device (CCD) camera. Image data captured with the CCD camera were fed into a desktop digital computer (PC-9801 BA2; NEC Corp., Tokyo, Japan) using a scanner (Image Scanner HS60F; Omron, Tokyo, Japan), and then analyzed with a self-made computer program developed for data processing.

Determination of the Solubility of MET

An excess amount of MET was dissolved in 40% ethanol solution and incubated with shaking in a water bath (37°C) for 24 h. The supernatant solution was then removed and the sample was obtained from the residual solution. MET concentration was determined using GC (GC-17A; Shimadzu Corp.). The column employed was a SAC-5 (15 m length × 0.25 mm; Supelco, Tokyo, Japan). The column, injection, and detector temperatures were maintained at 90, 200, and 90°C, respectively. Nitrogen was used as the carrier gas; the flow rate of nitrogen was maintained at 50 mL/min.

Determination of Relative Height Between Keratinocytes and the Intercellular Space

From the images of the CCD camera, the difference in relative height between keratinocytes and intercellular space was determined as follows:

$$\Delta H = H_{\text{keratinocytes}} - H_{\text{intercellular space}}$$

where ΔH represents the difference in relative height, $H_{\text{keratinocytes}}$ represents the height of the most raised (or dented) region of keratinocytes, and $H_{\text{intercellular space}}$ represents the height of the intercellular space. When ΔH has a positive value, it means that the keratinocytes were raised compared to the intercellular region, and a negative value means that the intercellular region was compared to the keratinocytes.

Lipid Extraction of the Stratum Corneum

The stratum corneum was separated from the excised dorsal skin of the hairless rat by floating the skin on the trypsin solution (0.1% in PBS, 24 h, 37°C). Washed and dried stratum corneum was put into the donor solution of the permeation study for 8 h. After that, the amount of ceramide or cholesterol that leaked into the donor solution was determined. Cholesterol was determined with a Cholesterol CII test Wako, whereas ceramide was determined by the method described by Lauter and Trams (9).

Preparation of Stratum Corneum Lipid Liposomes (SCLL)

SCLL was prepared via a hydration method (10). In brief, ceramide (40%), cholesterol (25%), palmitic acid (25%), cholesterol-3-sulfate (10%) were dissolved in a chloroform/methanol (2:1 in volume) mixture. The lipid mixtures were dried under a stream of nitrogen and then under vacuum at room temperature. Aqueous dispersions of the lipid mixtures were prepared by adding PBS and hydrating for 30 min. The suspensions were sonicated for 10 min at 80°C. The total lipid concentration of SCLL was approximately 5 mM.

Determination of Fluorescence Anisotropy

DPH was used as a probe in determining fluorescence anisotropy. The fluorescence anisotropy of the fluorescent lipophilic probe, DPH, embedded in the lipid bilayers of

SCLL, represents the degree of structural order in the lipophilic bilayer regions. DPH dissolved in *N*,*N*-dimethylformamide was added to the diluted SCLL solutions (2.5 mM lipid) in PBS. The final DPH concentration in the solution was 1.0 μM. The solutions were incubated with shaking at 37°C in the dark for 2 h, and the labeled SCLL was prepared. Each of the enhancers (1.0 mL) was mixed with the labeled SCLL and incubated with shaking at 37°C in the dark for 2 h. The fluorescence anisotropy of DPH was measured by using a fluorescent spectrometer (Hitachi F-4010; Hitachi, Tokyo, Japan) equipped with polarizer and thermoregulated cells at 37°C. The excitation wavelength was 360 nm and the emission wavelength was 430 nm for DPH. The degree of anisotropy (A) was calculated using the following equation:

$$A = (I_{//} - I_{\perp})/(I_{//} + 2I_{\perp})$$

where $I_{//}$ and $I_{\perp}$ refer to the horizontal and vertical orientations of fluorescence, respectively.

Statistical Analysis

Statistical analysis (one-way ANOVA) was performed using STATISTICA (StatSoft, Inc., Tucsa, OK, USA).

RESULTS AND DISCUSSION

Visualization of the Promoting Activity of MET on the Distribution of Fluorescent Probes in the Stratum Corneum

Laser scanning confocal microscopy (LSCM) has been employed to investigate the skin permeation of drugs (11–15). It is possible to observe an arbitrary point in the sample three-dimensionally; thus, a clear tomography of the skin surface was obtained by using LSCM. For further advantages of using this technique, refer to (16). In this study, LSCM was used to clarify MET's site of action in the stratum corneum. The chemical structures of the fluorescent probes used in this study are shown in Fig. 1.

The distribution of fluorescein in the stratum corneum is shown in Fig. 2. In the control (without ethanol and MET), fluorescein solution was administered to the skin surface for 8 h. However, fluorescence was not absolutely observed on and inside the stratum corneum. It was suggested that fluorescein in the donor solution was not able to move into the stratum corneum from the aqueous solution. In the case of treatment with 40% ethanol solution of fluorescein, the shape of the keratinocytes was recognized at the surface of the stratum corneum. Furthermore, fluorescein was also distributed in the keratinocytes and around the hair follicles. This means that ethanol enhances the distribution of fluorescein to the skin surface. The enhancement effect of ethanol was interpreted to be an increase in fluidity of the lipid domain in the stratum corneum or the alteration of the permeation pathway of drugs (17,18). In the case of diclofenac, ethanol contributed to the increase in solubility, and hence the flux of diclofenac increased (19). However, the distribution of fluorescein was only in the surface of the stratum corneum. At 12 μm below the surface, fluorescence was scarcely observed.

Furthermore, the distribution of fluorescein was remarkably increased in the intercellular space and keraninocytes region by the addition of MET to the donor solution compared to control or treatment with 40% ethanol solution. It was suggested that MET enhanced the distribution of fluorescent probes to the stratum corneum. Moreover, fluorescence was detected in the deeper skin tissues following the application of MET. It was considered that MET affected

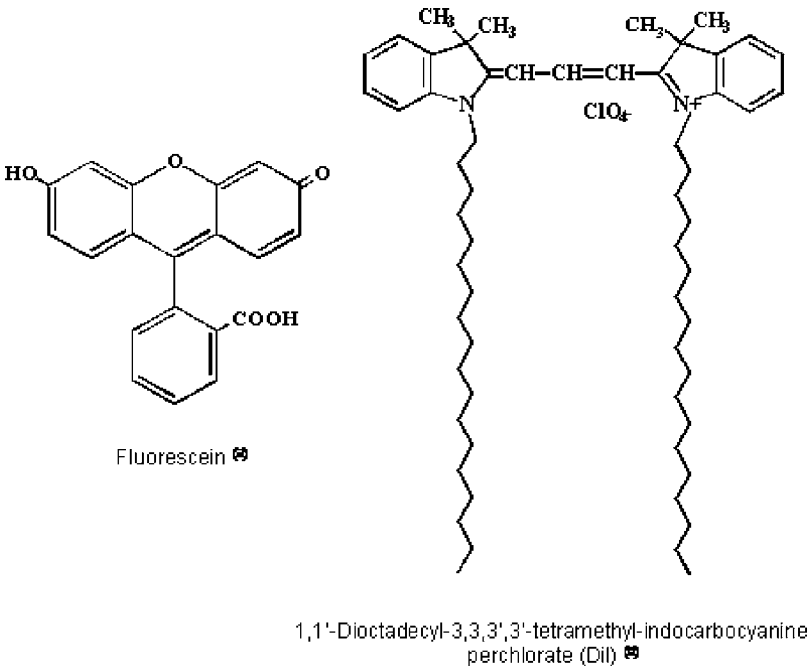

Fig. 1. Chemical structures of the fluorescent probes used in this study. (a) Hydrophilic probe. (b) Lipophilic probe.

deeper skin tissues compared to ethanol. In our previous study, it was reported that MET increased both partition and diffusion of ketoprofen (6). Thus, it was considered that MET contributed to the enhancement of the partition and diffusion of fluorescein, and to deliver greater amounts and penetrate deeper regions. We have also reported that terpenes needed to coexist with ethanol to exhibit their promoting activity (19). In the case of MET, it was also thought that the presence of ethanol helped the delivery of MET to deeper skin tissues.

Subsequently, the effect of MET on lipophilic fluorescent probe was evaluated. In the case of DiI (Fig. 3), fluorescence was distributed in the intercellular space in the control. This means that the hydrophobic probe distributed easily in the lipophilic region of the skin surface compared to hydrophilic probes. In the case of the application of 40% ethanol, the amount of distributed fluorescence increased. Furthermore, the application of MET resulted in the delivery of the fluorescent probe to deeper skin tissues. In this study, DMSO was used to dissolve DiI in the donor solution. It has been generally reported that DMSO has an enhancing effect on the skin permeation of drugs (20). However, a relatively greater amount (>20%) of DMSO is necessary to enhance the permeability of drugs. It was considered that 0.1% DMSO is not enough to affect the distribution kinetics of DiI to the skin surface.

From these results, it was suggested that the distribution of both hydrophilic and hydrophobic fluorescent probes was increased by the application of ethanol and MET. Turner and Guy (12) reported that the hydrophilic fluorescent probe distributed around the hair follicle. Thus, a difference was revealed in the promoting mechanisms between chemical enhancers such as MET and iontophoresis. Moreover, it was clearly recognized that LSCM is a very useful tool to visualize the promoting activity of chemical enhancers. However, when LSCM is used for mechanistic study, experimental conditions, including the characteristics of probes or direction of donor solutions, should be carefully defined.

Effect of MET on Skin Surface Morphology Observed Using Laser Scanning Microscopy

We have already reported that morphological changes were induced on skin surface due to the administration of menthol or MET (21). Based on scanning electron microscopy (SEM) results, the intercellular space was enlarged and the shape of the keratinocytes became easily recognized due to the administration of MET.

Laser scanning microscopy was used to clarify the quantification of morphological change. Laser scanning microscopy, which has an optical system, performs better compared to conventional optical microscopy. Furthermore, it is possible to obtain quantitative information precisely and noninvasively from the skin surface. Based on these advantages, we determined the surface morphology of the skin after MET was applied. Original and confocal images are shown in Fig. 4. In the confocal images, the white part indicates the relatively raised region. In the control and the skin treated with 40% ethanol for 8 h, the keratinocytes were raised compared to the intercellular region. On the other

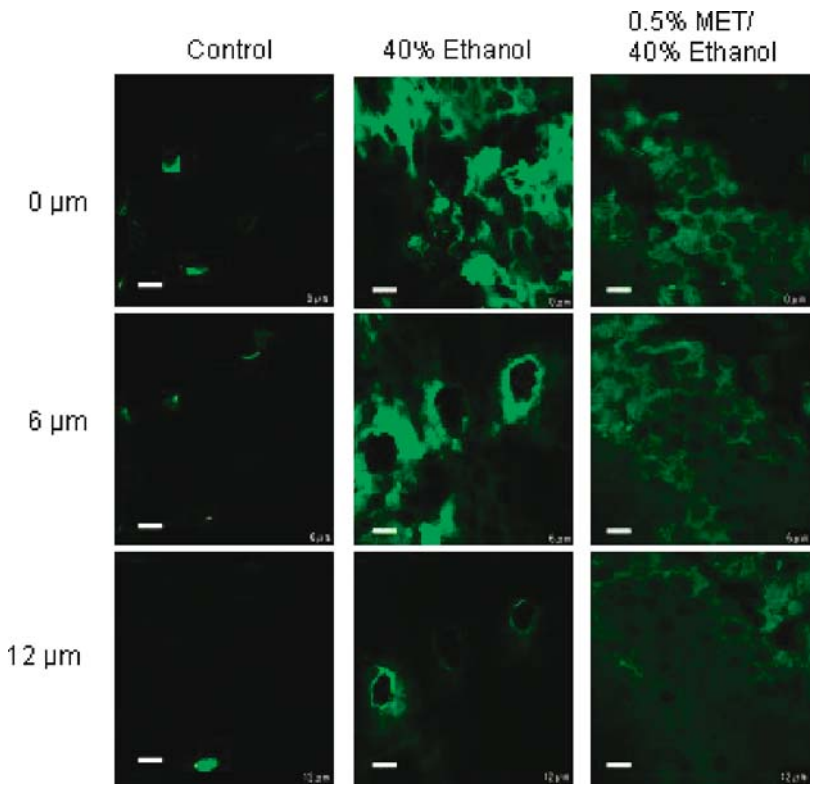

Fig. 2. Confocal fluorescence images of hairless mouse skin administered with fluorescein for 8 h. Bar = 5 μm.

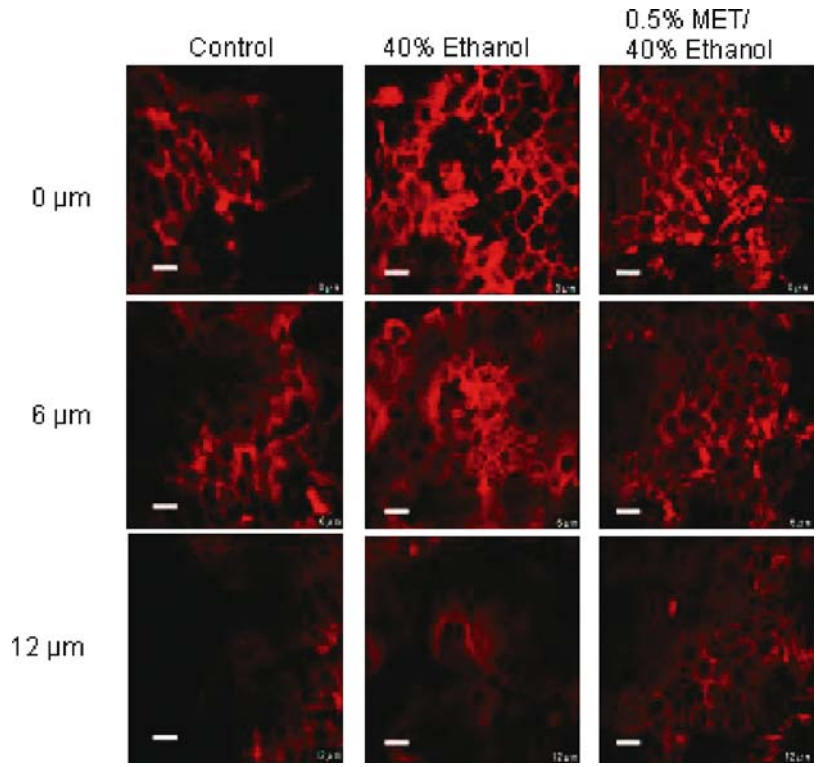

Fig. 3. Confocal fluorescence images of hairless mouse skin administered DiI for 8 h. Bar = 5 μm.

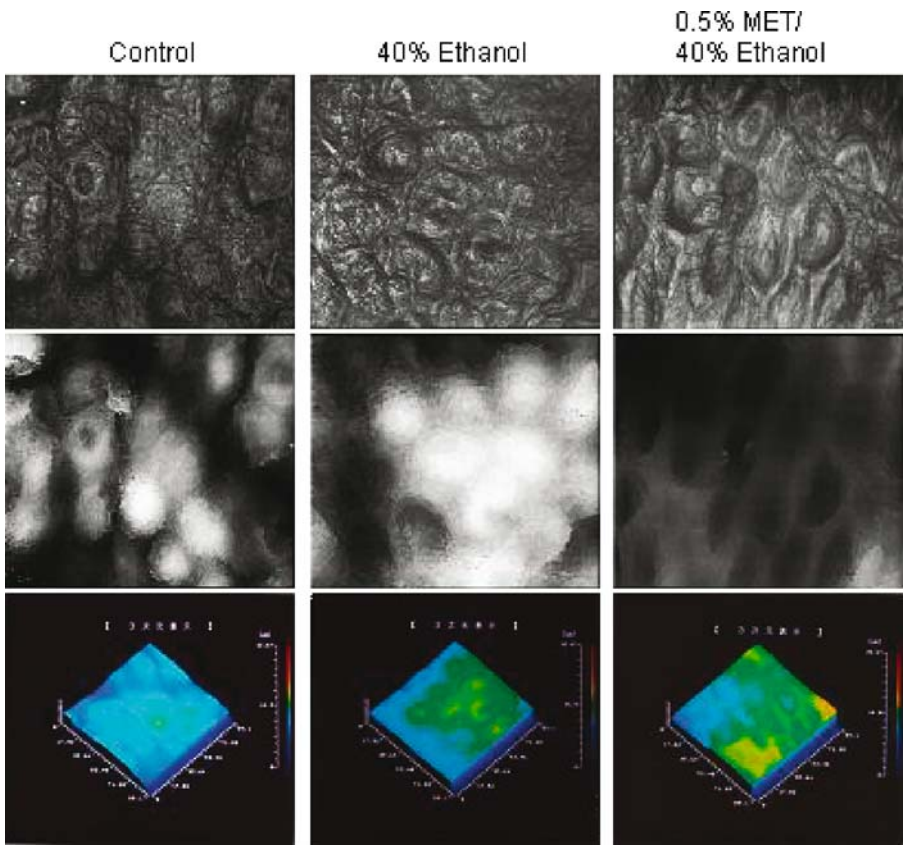

Fig. 4. Images of the skin surface of a hairless mouse using the laser scanning microscope.

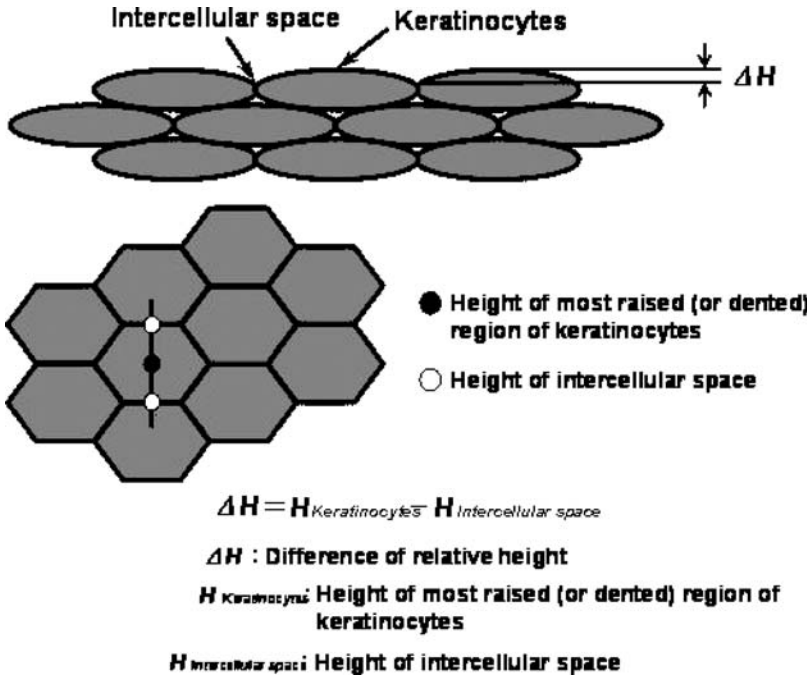

Fig. 5. Schematic diagram of the evaluation of skin surface morphology.

hand, following the administration of 0.5% MET, the intercellular region was raised compared to keratinocytes. It was suggested that MET acted on the intercellular region of the stratum corneum. Relative height in the stratum corneum was quantified by using the data from confocal images. Details of the quantification are schematically represented in Fig. 5. The difference in height between the keratinocytes and intercellular space (ΔH) was defined as follows:

$$\Delta H = H_{\text{keratinocytes}} - H_{\text{intercellular space}}$$

where $H_{\text{keratinocytes}}$ is the height of the center of keratinocytes and $H_{\text{intercellular space}}$ is the height of the intercellular space. As shown in Fig. 6, positive ΔH values were observed following the administration of water or 40% ethanol for 8 h. It was considered that the keratinocytes were hydrated by treatment with water or 40% ethanol. Furthermore, 40% ethanol treatment affected the intercellular space; consequently, the ΔH value slightly decreased compared to that of the control. On the other hand, when MET was administered to the skin surface, the increase in height of the intercellular space exceeded that of the keratinocytes. Thus, negative values of ΔH were observed as a result of MET treatment. This means that the intercellular region was swollen by treatment with MET. Moreover, ΔH values significantly decreased when the amount of MET applied was increased. It was suggested that MET acted in the intercellular regions and changed their structure. The increase in MET in the donor solution caused further expansion of the intercellular space. However, no further decrease in the ΔH value was observed due to the increase in the additive concentration of MET to the donor solution exceeding a concentration of 0.5%. From the results of the determination of solubility, it was clarified that MET can be dissolved 0.36% in 40% ethanol. This value is thought to be acceptable when the lipophilicity of the compound is taken into consideration (4). This means that the thermodynamic activity of MET in the donor solution was already maximized and no more molecules moved to the skin surface. This result coincided well

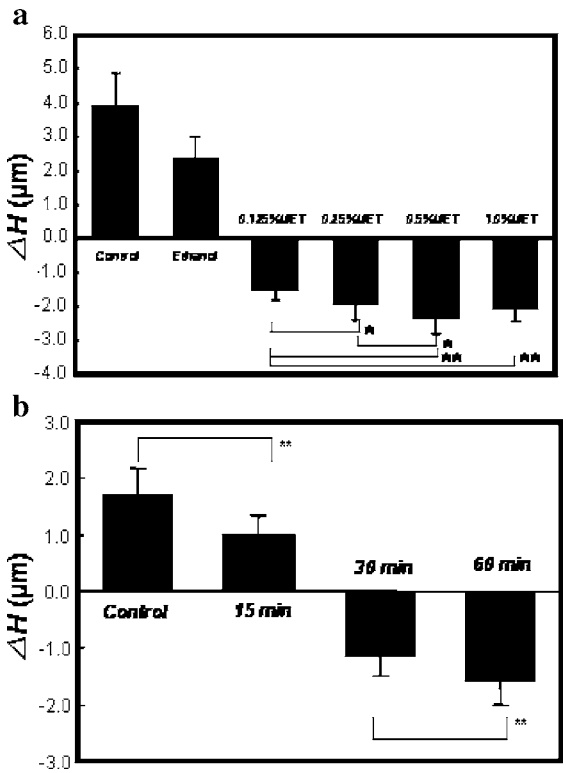

Fig. 6. Effect of the concentration of MET on the skin surface morphology of the hairless mouse dorsal skin 8 h after its administration. Each value represents the mean ± SD (n = 10–15), $^{*}p < 0.05$, $^{**}p < 0.01$.

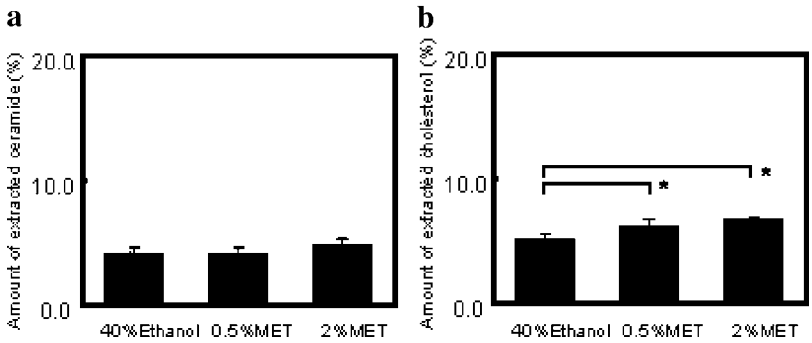

Fig. 7. Effect of MET on the extraction of ceramide or cholesterol from the stratum corneum of the hairless rat incubated for 2 h. Each value represents the mean ± SD ($n = 4$), $*p < 0.05$.

with the fact that the flux of oxybutinyn was maximized at around 0.5% of MET and no further increase in flux was observed (22).

Subsequently, the administration period was evaluated for 0.5% MET. In Fig. 6, "control" means the skin was fixed in 8% formaldehyde–buffer solution just after being excised. ΔH was significantly decreased by the increase in treatment time. Furthermore, ΔH value shifted to a negative value after 30 min of MET administration. This result suggested that keratinocytes are ordinarily raised compared to intercellular space. However, it was clarified that the rigid structure of intercellular space was swollen by MET, ethanol, and water just after administration. A remarkable change in the stratum corneum structure was brought about by the 30-min MET administration because of the negative value of ΔH. It was considered that MET required only a short length of time to exhibit its promoting activity, which changed the dense packing of the intercellular lipid bilayer. The change in skin surface caused by the administration of MET became moderate after 1 h. The effect of *d*-limonene or *l*-menthol on skin permeation of diclofenac was maximized after only 1 h of pretreatment (5). No further increase in permeability coefficient was observed with the increase in pretreatment period. Thus, from these results, it was suggested that the remarkable change in skin surface structure coincided well with the promoting activity. Furthermore, the change necessary to exhibit promoting activity was induced within 30 min after administration.

Effect of MET on Lipid Extraction of the Stratum Corneum

The active site of MET was thought to be the intercellular lipid domain based on the results of the morphological change in intercellular space. The lipid composition of the intercellular space was reported to be ceramide (45–50%) and cholesterol (30–40% including esters). Thus, the affinity of MET to those lipid components was investigated. In general, lipid extraction was considered to be one of the mechanisms of several percutaneous absorption enhancers (20). In this study, the extraction of ceramides and cholesterol from the stratum corneum was evaluated. The ratio of the extracted amount against the amount extracted via the chloroform/methanol (2:1) mixture was defined as an index for the evaluation of lipid extraction. As shown in Fig. 7, the amount of ceramides leaked into the donor solution was almost the same as that of the control or 40% ethanol, independent of the concentration of MET. It is well known that the barrier function of the skin is caused by the function of ceramides in the intercellular space. Because MET showed little effect on the extraction of ceramides, it means that MET does not diminish the essential barrier function of stratum corneum to prevent dehydration and invasion of foreign substances. On the other hand, the amount of cholesterol leaked was significantly increased by the administration of MET compared to that of 40% ethanol. Cholesterol is widely recognized to stabilize the lipid bilayer. Thus, the tightly packed lipid bilayer of the stratum corneum

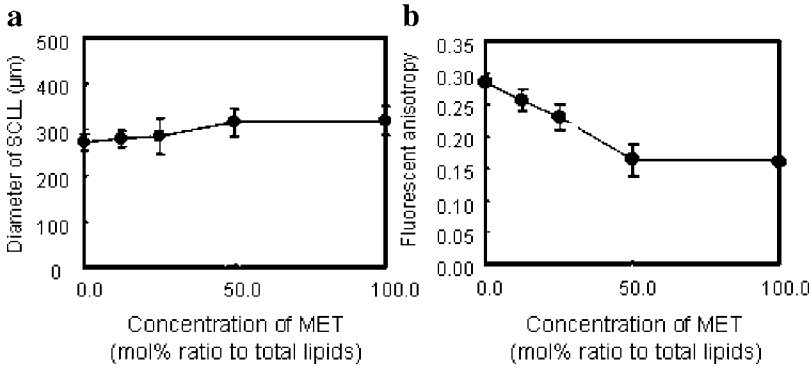

Fig. 8. Effect of MET on the diameter or fluorescence anisotropy of DPH in SCLL. Each value represents the mean ± SD ($n = 4$).

was loosened by the extraction of cholesterol. Consequently, the fluorescent probes easily distributed to deeper regions and the intercellular space was swollen due to MET treatment.

Effect of MET on the Fluidity of the Liposome Membrane

From the results of the quantification of the surface morphology of the stratum corneum, it was revealed that MET treatment expanded the intercellular space. Subsequently, to perform a more precise investigation, stratum corneum lipid liposomes were used as a model of the intercellular lipid domain. It is widely known that the intercellular space of the stratum corneum consists of a lipid bilayer. In the field of transdermal drug delivery, liposomes are often used to determine the mechanism of absorption enhancers. In this study, liposomes containing ceramide, cholesterol, palmitic acid, and cholesterol-3-sulfate were selected as a simple model for the intercellular lipid bilayer of the stratum corneum (10,23,24). MET was dissolved in 40% ethanol and added to the liposome solution just before incubation (2 h). As shown in Fig. 8a, the size of liposome was significantly increased by the administration of MET [$F_{(4,40)} = 5.0894$, $p < 0.005$]. It was suggested that MET significantly increased the fluidity of lipids in the liposome membrane depending on the dose level. In the case of the administration of PBS, the average diameter of the liposomes was 266.6 ± 24.3 μm. On the other hand, when 40% ethanol was administered to the liposomes, the diameter was 272.7 ± 24.3 μm. The change in diameter was insignificant following the administration of 40% ethanol. Furthermore, the fluorescence anisotropy of DPH was significantly decreased with the increase in the additive concentration of MET [$F_{(4,5)} = 46.477$, $p < 0.01$]. There was also evidence of an increase in the fluidity of the liposome membrane. From these results, it was suggested that MET contributed to the enhancement of the perturbation of lipid bilayer, and thus the diffusivity of drugs in the lipid bilayer in the stratum corneum increased.

CONCLUSIONS

Based on results, MET enhanced the distribution of drugs to the intercellular region of the stratum corneum. In the confocal images of the skin surface, the change in structure of the intercellular region was clearly demonstrated by MET treatment. Furthermore, MET increased the lipid fluidity of the liposome membranes. It was suggested that MET contributed to the enhancement of both the partition and diffusion of drugs, and hence promoted the percutaneous absorption of the drugs.

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