Effects of $l$-menthol on the Thermotropic Characteristics of Intercellular Lipid in the Hairless Rat Stratum Corneum Evaluated by Differential Scanning Calorimetry and Electron Spin Resonance

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Transdermal absorption enhancers (e.g., $l$-menthol) act on intercellular lipid in the stratum corneum (SC), although the detailed mechanism of action of $l$-menthol on intercellular lipid is unclear. Using differential scanning calorimetry and electron spin resonance (ESR) to evaluate the promoting mechanisms of $l$-menthol, we focused on the effects of $l$-menthol on the microviscosity of intercellular lipid in the SC. Treatment with $l$-menthol significantly decreased the transition temperatures of the microstructure of intercellular lipid in the SC. ESR measurements using two kinds of spin-label reagent, 5- and 16-doxyl stearic acid (5- and 16-DSA) showed that $l$-menthol at physiological temperatures decreased the rotational correlation time ($\tau$) of 5-DSA and that the flexion point shifted to the lower temperature. $l$-Menthol decreased the $\tau$ value of 16-DSA at ambient temperature. These data suggest that $l$-menthol reduces the microviscosity of the hydrophobic region in intercellular lipid in the SC, an effect caused by the disruption of the microstructure of intercellular lipid. In conclusion, $l$-menthol fluidizes the polar and hydrophobic region of intercellular lipid in the SC. These data might provide useful information for the development of effective transdermal formulations.

Introduction

The stratum corneum (SC), the outermost layer of the skin, performs a vital function as a barrier against foreign stimuli e.g., viruses, bacteria, ultraviolet light, and drying. The SC structure can be described by the “bricks and mortar” model, in which corneocytes are embedded in a lamellar lipid matrix [1]. The corneocytes correspond to the bricks and lipids act as mortar to fill the intercellular space. Even though it comprises only a small portion of the SC, intercellular lipid plays an important role by providing the barrier function of the skin. The major components of intercellular lipid in the SC are ceramides, cholesterol, free fatty acids and these derivatives of three components [2,3]. Ceramides compose about 50% of the total intercellular lipid mass. Human SC includes 11 structurally heterogeneous types of ceramides, termed ceramide 1 to 11 [4]. Cholesterol comprises about 25% and fatty acids comprise about 10% of the total lipid mass in the SC.

The lipid microstructure and the molecular arrangement of intercellular lipid in the SC has been investigated using synchrotron X-ray scattering and neutron diffraction [5-7]. Two kinds of lamellar structure are observed by small-angle X-ray scattering and neutron diffraction. One is a short lamellar structure with a repeat distance of about 6 nm, and the other is a long lamellar structure with a repeat distance thought to be about 13 nm [6,8]. Hydrocarbon chain packing is observed by wide-angle X-ray scattering, and a lattice distance of about 0.42 nm and orthorhombic packing with lattice distances of about 0.42 and 0.37 nm have been observed [9]. These microstructures of intercellular lipid in the SC relate to the barrier function of the skin. To improve drug absorption via the skin, it is necessary to disturb gently the microstructures of intercellular lipids in the SC.

The most widely used approach to improve transdermal drug absorption involves chemical absorption enhancers, including the cyclic monoterpenes [10-13]. Although the mechanism responsible for this enhancement effect has not been clarified, the fluidizing effect of cyclic monoterpenes on intercellular lipid in the SC is thought to be a crucial factor [14,15]. We used Fourier transform infrared spectroscopy to observe the conformational disorder of intercellular lipids in the SC induced by treatment...
with l-menthol, one of the most commonly used cyclic monoterpenes [16]. Using electron spin resonance (ESR) spectroscopy, Anjos et al. also reported that terpenes, including l-menthol, disrupt the hydrogen bond network of the polar head groups in intercellular lipid in the SC [17,18]. Understanding this effect of l-menthol at the molecular and structural levels requires further study.

ESR spectroscopy is a useful methodology for evaluating the microviscosity of intercellular lipids in the SC and l-menthol-induced changes in intercellular lipid at the molecular level. The ESR spectrum of spin-labeled fatty acids is highly sensitive to the motional properties of the spin-label reagents, permitting one to monitor the changes that occur in intercellular lipid in the SC in response to added drugs [19-21]. The ESR technique provides an efficient tool for distinguishing the fluidizing effect of transdermal absorption enhancers. We used differential scanning calorimetry (DSC) and ESR measurements to investigate the effects of l-menthol on the thermotropic behavior of the SC and on the hydrophobic and polar domains of intercellular lipid in the SC.

**Experimental**

**Chemicals**

l-Menthol was purchased from Tokyo Chemical Industries Co. Ltd. (Tokyo, Japan). Ethanol and trypsin were purchased from Wako Pure Chemical (Tokyo, Japan). The spin-labeled derivatives of stearic acid, 5- and 16-doxyl stearic acid (5-DSA and 16-DSA), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals used were reagent grade.

**Preparation of the SC sheet**

The hairless rat SC was separated from the excised abdominal region of hairless rats (HWY/Slc, 12 weeks old, Sankyo Labo Service, Tokyo, Japan) by digestion with 0.1% trypsin in phosphate-buffered saline (pH 7.4) at 37 °C for 24 h. The separated SC was rinsed in purified water and dried in vacuo. The procedures involving animals and their care complied with the regulations of the Committee on Ethics in the Care and Use of Laboratory Animals of Hoshi University.

**DSC measurements**

The SC was incubated in purified water or penetration enhancer solution (40% ethanol, 1% l-menthol in 40% ethanol) for 2 h at ambient temperature and dried under a stream of nitrogen until it reached an acceptable predetermined weight (125% of the weight before treatment). DSC measurements were performed using a Thermo plus DSC-8230 instrument (Rigaku Co., Tokyo, Japan) with heating scans at the rate of 10 °C/min. The samples were placed in an aluminum pan (Rigaku Co.). The transition temperature was determined as the peak of the endothermic transition profiles.

**ESR measurements**

The dried SC sheet was incubated in 10 mM spin-label reagent (5- or 16-DSA) having the nitroxide radical moiety (doxyl) in the 5th or 16th carbon atom of the acyl chain, in 40% ethanol or 1% l-menthol in 40% ethanol solution for 2 h at 37 °C. After the spin labeling, the excess spin label was washed out using 40% ethanol solution. The prepared SC sheet was introduced into the capillary tube for the ESR measurements.

ESR measurements were performed using a JEOL JES-FA200 spectrometer (JEOL, Tokyo, Japan). ESR measurement conditions were microwave frequency of 9.07 GHz, microwave power of 5.0 mW, modulation width of 1.2 × 0.1 mT, magnetic field of 325 ± 5 mT, sweep time of 30 s and time constant of 30 ms. The temperature of the SC was controlled from 20 to 70 °C at the rate of about 2.5 °C/min using ES-DVT4. The rotational correlation time τ (ns) was calculated from the obtained ESR spectra based on equation appeared in the reference [22].

**Statistical analysis**

Each value is expressed as the mean ± S.D. For group comparisons, a one-way layout analysis of variance (ANOVA) and Student’s t test were used to analyze the data. P values less than 0.05 were considered significant.

**Results**

**Effects of l-menthol on the thermotropic behavior of the hairless rat SC**

The distinct changes in the thermotropic behavior of the hairless rat SC induced by the treatment with l-menthol are shown in Fig. 1. The intact SC showed three endothermic phase transitions near 39 °C, 54 °C, and 66 °C (Fig. 1(a)). These endothermic peaks in the profiles were shifted to a lower temperature by treatment with the 40% ethanol and 1% l-menthol in 40% ethanol solution. Fig. 2 shows the phase transition temperatures obtained from the thermograms in Fig. 1. These phase transition temperatures were decreased significantly by the treatment with 1% l-menthol in 40% ethanol, from 39 °C, 54 °C, and 66 °C to 35 °C, 48 °C, and 58 °C, respectively. By contrast, treatment with 40% ethanol decreased the phase transition temperature insignificantly.

**Effects of l-menthol on the microviscosity of intercellular lipid in the SC**

The ESR spectra of the spin-label reagent 5-DSA in intercellular lipid in the SC are depicted in Fig. 3. The spectra of 5-DSA in intercellular lipid in the SC indicated two distinct peaks derived from the S and W components. The component S decreased markedly as a function of temperature, whereas the effect of temperature on the component W was modest. As shown in Fig. 3(b), l-menthol strongly affected the component S in the spectra of 5-DSA. The component S was weakened significantly by treatment with 1% l-menthol in 40% ethanol. Fig. 4 shows the τ values calculated from the ESR spectra of 5-DSA in Fig. 3. Treatment with l-menthol markedly decreased the flexion point of the τ value of the SC.
Fig. 1  DSC thermograms of the hairless rat SC after treatment with l-menthol in 40% ethanol. (a) Purified water as a control, (b) 40% ethanol, and (c) 1.0% l-menthol in 40% ethanol were applied to the SC for 2 h at 37 °C.

Fig. 2  Changes in the phase transition temperature of the hairless rat SC. The phase transition temperatures were calculated from the DSC curves shown in Fig. 1. Open bars, control, purified water-treated SC; gray bars, 40% ethanol; and black bars, 1.0% l-menthol. Ortho, orthorhombic hydrocarbon chain packing; LL, long lamellar structure; SL, short lamellar structure; and H-Hex, high-temperature hexagonal hydrocarbon chain packing. Each column represents the mean ± S.D. (n=3). *p<0.05 and **p<0.01 vs. control.

Fig. 3  ESR spectra of 5-DSA in the hairless rat SC after the treatment with l-menthol in 40% ethanol. (a) 40% ethanol and (b) 1.0% l-menthol in 40% ethanol were applied to the SC for 2 h at 37 °C. The ESR spectra were obtained under thermal control at a rate of about 2.5 °C/min.
Fig. 4  Effects of l-menthol on the fluidity of the hydrophilic region of intercellular lipid in the SC. The rotational correlation times of 5-DSA in the hairless rat SC were calculated from the ESR spectra shown in Fig. 3. (○) Control, 40% ethanol-treated SC and (□) SC treated with 1.0% l-menthol in 40% ethanol.

Fig. 5  ESR spectra of 16-DSA in the hairless rat SC after treatment with l-menthol in 40% ethanol. (a) 40% ethanol and (b) 1.0% l-menthol in 40% ethanol were applied to the SC for 2 h at 37 °C. The ESR spectra were obtained under thermal control at a rate of about 2.5 °C/min.

Fig. 6  Effects of l-menthol on the fluidity of the hydrophobic region of intercellular lipid in the SC. The rotational correlation times of 16-DSA in the hairless rat SC were calculated from the ESR spectra shown in Fig. 5. (○) Control, 40% ethanol-treated SC and (□) SC treated with 1.0% l-menthol in 40% ethanol.
as the temperature decreased from 37.5 °C to 30 °C. The l-menthol in 40% ethanol treatment markedly decreased the τ value at room temperature, and the τ value decreased further at lower temperatures.

Figs. 5 and 6 show the effects of l-menthol on the ESR spectra and values of the spin-label reagent 16-DSA in intercellular lipid in the hairless rat SC. The ESR spectra of 16-DSA showed isotropic peaks, and these peaks sharpened gradually as a function of temperature. The τ values decreased gradually as the temperature rose from 20 °C to 60 °C. The τ value of 16-DSA was much lower in the 1.0% l-menthol-treated SC than in the 40% ethanol-treated SC. With increasing temperature, the τ value converged to that obtained for intercellular lipid in the SC treated with 40% ethanol at 60 °C.

Discussion

The effect of pharmaceutical additives, including transdermal absorption enhancers, on intercellular lipid in the SC is important to the development of effective transdermal drug delivery systems. The interaction between transdermal absorption enhancer molecules and components of intercellular lipid in the SC might be significant. The mechanistic alterations in the microstructure and physicochemical properties of intercellular lipid in the SC may be induced by this interaction. The enhancing effect of the cyclic monoterpenes is related to the lipid fluidity of intercellular lipid in the SC [16, 19]. Therefore, clarifying a drug's permeation route requires the evaluation of the fluidizing effect of the cyclic monoterpenes at the molecular level.

The thermograms of the SC indicated three peaks that were derived from endothermic phase transitions (Fig. 1(a)). Previous results show that these phase transitions reflect changes in intercellular lipid in the SC [23, 24]. A previous study using DSC reported that the phase transition of intercellular lipid in the hairless mouse SC occurred at 39 °C, 51 °C, and 71 °C [23]. The change in lamellar structure in intercellular lipid is consistent with the phase behavior investigated by synchrotron X-ray scattering in the hairless mouse SC [24]. Intercellular lipid in the SC of the hairless mouse is thought to form a rigid lamellar structure and to comprise hydrocarbon chain packing at temperatures < 39 °C. At 39 °C, the orthorhombic hydrocarbon chain packing disappears and is transposed to high-temperature hexagonal hydrocarbon chain packing at higher temperatures. The disappearance of the long lamellar structure occurred at 51 °C and the structure transferred to a liquid crystalline phase with hexagonal hydrocarbon chain packing. Subsequently, the short lamellar structure melted at 71 °C. It was reported that the thermotropic behavior of the hairless rat SC was similar to that of the hairless mouse [25]. Therefore, the obtained endothermic phase transition at 39 °C represents the disappearance of the orthorhombic hydrocarbon chain packing. Moreover, the phase transitions at 54 °C and 66 °C reflect the phase transitions of long and short lamellar structures, respectively. As shown in Fig. 2, l-menthol significantly decreased each phase transition temperature of intercellular lipid in the SC, suggesting that l-menthol disturbed and/or fluidized the rigid microstructure of intercellular lipid in the SC. We have previously used synchrotron X-ray scattering to show that l-menthol significantly affects the intercellular lipid arrangement of the hairless rat SC [16]. A similar effect was observed in a lipid model system [26]. These reports suggest that the change in intercellular lipid arrangements induced by treatment with l-menthol is a crucial factor in its enhancing effect. To correlate these thermotropic behaviors of intercellular lipid in the SC and lipid fluidity, we performed the ESR measurements according to the thermal control.

The spin-label reagents 5- and 16-DSA were used to detect the phase transitions [27, 28]. They reflect only the reorientation motion of the nitroxide radical, which depends greatly on the motional freedom of the alkyl chain to which it is bound (Fig. 7). In the case of 5-DSA,
the doxyl group is located near the carboxyl group, and the nitroxide radical should be accommodated near the head group, which is a polar region of the intercellular lipid lamellar matrix. In 16-DSA, the nitroxide radical is bound to the terminal methylene group, where it reflects the segmental motion of the alkyl chain, a hydrophobic region of the intercellular lipid lamellar matrix. Consequently, the ESR spectra obtained from these spin-label reagents can clarify the effects of l-menthol on different regions of intercellular lipid in the SC. The rotational correlation time (τ) is expressed in Eq. (1)[22].

\[ \tau = 3.418 \times 10^{-6} \Delta(0) \left[ \frac{h(0)}{q_n(1)} - \frac{h(1)}{q_n(1)} \right] \]  

\( \tau \) is rotational correlation time, \( \Delta(0) \) is the peak-to-peak width of the central line in Gauss, \( h(m) \) is the peak height of the line belonging to the nuclear quantum number \( m \). \( \tau \) represents micro viscosity around probes, such as 5- and 16-DSA and also inversely proportional to membrane fluidity.

The \( \tau \) value of 5-DSA decreased markedly as the temperature increased in 30 °C to 40 °C. This decrease was accompanied by distinct changes in the flexion point, which corresponded to the phase transition of the orthorhombic hydrocarbon chain packing (Fig. 1(a)). This decrease in the \( \tau \) value of 5-DSA suggested that fluidization of the polar region in the intercellular lamellar was dependent on the phase transition of the orthorhombic hydrocarbon chain packing. Therefore, the polar region of intercellular lipid in the SC was fluidized secondarily by disturbances in the hydrocarbon chain packing. In contrast, the \( \tau \) value of 16-DSA decreased gradually with increasing temperature, suggesting that the hydrophobic region of intercellular lipid in the SC fluidized gradually as a function of temperature. The conformational state of the hydrocarbon chain in ceramide, the main component of intercellular lipid in the SC, became disordered as a function of temperature. This was accompanied by conformational disorder of the hydrocarbon chain and the transition from the trans rotamer to the gauche structure, as shown by Raman spectroscopy [29, 30]. This disorganization was accompanied by a loosening of the lateral packing in the hydrocarbon chain packing. This disordering was induced by the loosened hydrogen bond and van der Waals forces of the lipid molecules [31]. These results suggest that the decrease in the \( \tau \) values of 16-DSA is related to the rotameric transition from the \textit{trans} to the \textit{gauche} structure of the hydrocarbon chain backbone.

l-Menthol reduced the \( \tau \) values of 5- and 16-DSA in intercellular lipid in the SC at the physiological temperature (Figs. 4 and 6), suggesting that l-menthol fluidized the polar and hydrophobic regions of intercellular lipid in the SC. In the experiment with 5-DSA, intercellular lipid in the SC showed a distinct flexion point, and l-menthol significantly shifted the flexion point from 35 °C to 27 °C. This flexion point corresponds to the phase transition of the orthorhombic hydrocarbon chain packing of intercellular lipid in the SC. These data suggest that the intermolecular interaction with the orthorhombic hydrocarbon chain packing of intercellular lipid in the SC was weakened by treatment with l-menthol at the physical temperature. We suggested previously that l-menthol significantly fluidizes the hydrocarbon chain packing of intercellular lipid in the SC based on our data from synchrotron X-ray scattering [15]. Our current data clarify that the fluidizing effect of l-menthol on intercellular lipid in the SC was induced by disturbances in the hydrocarbon chain packing in intercellular lipid model systems comprising ceramide 5, cholesterol, and palmitic acid [26]. These data suggest that fluidization of the polar region of intercellular lipid in the SC was caused by attenuation of the intermolecular interaction of the head groups.

In experiments with the other spin-label reagent, 16-DSA, the \( \tau \) values were decreased markedly by treatment with l-menthol at lower temperatures, suggesting fluidization of the hydrophobic region of intercellular lipid in the SC, which comprises mainly a hydrocarbon backbone of ceramide. l-Menthol may affect the rotameric conformation of the hydrocarbon chain of intercellular lipid in the SC by inducing a transition from the \textit{trans} to \textit{gauche} rotamer in the hydrocarbon chains. Another report suggested that l-menthol attracts membrane lipids to the center of the membrane, making the interior of the membrane more polar, which would disturb the hydrogen-bonding network in the polar region of intercellular lipid in the SC [30]. Based on their ESR measurement data, Anjos et al. suggested that monoterpenes act mainly at polar head groups where they weaken the inter- and intralamellar hydrogen bonding networks [18]. Therefore, the effect of l-menthol on the hydrophobic region might be a subsequent action induced by the disruption of the polar hydrocarbon, hexagonal, and orthorhombic hydrocarbon chain packing. The ESR measurements suggested that the fluidizing effect of l-menthol reflects the thermotropic behavior, disturbance of hydrocarbon chain packing, and rotameric disorganization of intercellular lipid in the SC.

**Conclusions**

ESR is a powerful tool for evaluating the promoting activity of transdermal absorption enhancers and provides insight into the molecular and functional groups in intercellular lipid in the SC. From our results, we concluded that l-menthol acts on the polar and hydrophobic regions of intercellular lipid in the SC. A fluidizing effect was induced by changes in the microstructure of intercellular lipid in the SC. The results of this study may provide important knowledge for the development of transdermal formulations.

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**References**


