Selective Recovery of Deranged Water-Holding Properties by Stratum Corneum Lipids

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Selective removal of stratum corneum lipids following applications of acetone/ether to the human forearm for extremely prolonged periods of 5–20 min induced an enduring (more than 4 days), chapped and scaly appearance of the skin which was accompanied by a significant decrease in the water-holding properties of the stratum corneum. In order to further elucidate the significance of lipids in the water-holding properties, lipids, which were extracted as sebaceous-rich lipids (SL) for the first 10-min acetone/ether treatment and as stratum corneum lipids (SCL) for the additional 30-min treatment, were topically applied daily on lipid-depleted forearm skin which had been pretreated with acetone/ether for 40 min. Two daily applications of the SCL which were solubilized in squalane containing 1% α-monomethyl heptadecyl glyceryl ether (GE) caused a significant increase of conductance, accompanied by a marked improvement in the level of scaling as compared with non-treatment or GE/squalane base, whereas the SL in the GE/squalane base did not exhibit any significant recovery in either conductance value or scaling. To clarify which components of the SCL are primarily responsible for the observed recovery of the water-holding properties, chromatographically separated fractions of the SCL were also topically applied in the same manner for 2 successive days. Out of the following separated fractions: cholesterol, cholesterol ester, free fatty acid, glycolipids, and ceramide, 2 daily topical applications of ceramide fraction induced a significant and the highest increase in the conductance value as compared with GE/squalane base. Furthermore, glycolipids and cholesterol fractions also exhibited a significant recovery when compared with no application at all. In contrast, free fatty acid and cholesterol ester fractions did not indicate any significant increase in the conductance value. These findings strengthen the hypothesis that structural lipids present in the intercellular spaces of the stratum corneum, especially ceramide, play a critical role in the water-holding properties of the stratum corneum. *J Invest Dermatol 87:758–761, 1986*

Mammalian stratum corneum serves as a barrier against excess body water loss and cutaneous permeability whose functions have been suggested by Elias [1,2] to be attributable to the stratum corneum lipids (SCL). These barrier functions are also known to be impaired in the excess dry conditions which can be seen after solvent or surfactant treatment [3,4]. We have previously shown that application of acetone/ether to human skin for an extremely prolonged period of 20 min, as compared with the usual procedure for the extraction of sebaceous lipid on the skin surface, induced a chapped and scaly appearance of the stratum corneum which persisted at least until day 4 after treatment, despite no release of any hygroscopic materials such as free amino acids [5]. These impairments show a marked decrease in the water-holding capacity of the stratum corneum accompanied by a considerable and selective loss of intercellular lipids such as cholesterol, cholesterol esters, and polar lipids. In contrast, such a significant and persistent decrease in the water-holding capacity could not be induced after a short treatment period in which sebaceous gland lipids such as squalene, triglycerides, and wax esters could be predominantly extracted. Thus, it has been suggested that lipids which construct lamellar structures in the intercellular spaces of the stratum corneum could be a specific modulator for water-holding properties of the stratum corneum.

Because it is well established that SCL comprise several components such as cholesterol, glycosylsphingolipids, and fatty acid [6] which themselves possess no substantial capacity for holding water in extracted in vitro situations, it seems reasonable to assume that these lipids are specifically compartmentalized into the intercellular spaces to exert their water-holding properties. This led us to investigate which lipid components are primarily responsible for their water-holding properties. Since there is no available technique of selectively extracting certain lipid components alone from the stratum corneum in vivo, we have tried to measure the recovery potential of an extracted lipid or its chromatographic subfractions for the water-holding property after application on the lipid-depleted stratum corneum in which a marked decrease in the water-holding properties is found. In this paper, we report a selective improvement of the deranged water-holding capacity by ceramide fraction.

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Abbreviations:
GE: α-monomethyl heptadecyl glyceryl ether
SCL: stratum corneum lipid(s)
SL: sebaceous-rich lipid(s)
TLC: thin-layer silica gel chromatogram(s)

MATERIALS AND METHODS

Treatment with Acetone/Ether The forearm skin of 10 healthy male volunteers, aged 24–33 years, was used. Open-end, 3 cm-diameter cylinders filled with 10 ml of acetone/ether (1/1) were

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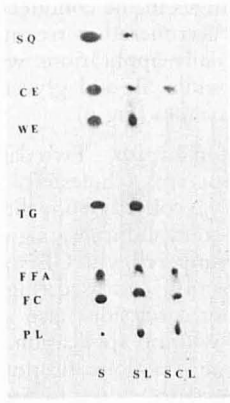

Figure 1. Thin-layer chromatographic plate sprayed with sulfonic acid. The 10-min extraction as SL and the additional 30-min extraction as SCL were analyzed with TLC using the first developing solvent (benzene:hexane, 1:1) and the second developing solvent (hexane:diethyl ether:glacial acetic acid, 70:30:1). *S*, standard; *SQ*, squalene; *CE*, cholesterol ester; *WE*, wax ester; *TG*, triglyceride; *FFA*, free fatty acid; *FC*, free cholesterol; *PL*, polar lipid; *SL*, sebaceous-rich lipid; *SCL*, stratum corneum lipids.

gently pressed with occasional shaking onto the sample areas for 10-min intervals to obtain a sebaceous-rich lipid (SL). Additional treatment for another 30 min was carried out to obtain a SCL fraction. Under these conditions the obtained SCL contained 10% cholesterol ester, 20% free fatty acid, 15% cholesterol, 50% ceramide, and 5% glycolipid in their lipid composition without contamination of sebaceous lipids, as analyzed by thin-layer silica gel chromatograms (TLC) which will be described later. For collection of a large amount of the SCL fraction, the forearm skin of 100 volunteers was used to obtain about 6–8 mg SCL/person from 8–10 areas (90–140 $\mu g/cm^2$).

Analysis and Separation of Lipids The 10-min acetone/ether extraction as SL and the additional 30-min extraction as SCL were analyzed by using one-dimensional thin-layer silica gel (Wako B5 gel, Wako Chemical Co., Japan) chromatograms, ultilizing benzene:hexane (1:1) as the first developing solvent and hexane:diethyl ether:glacial acetic acid (70:30:1) as the second (Fig 1). The extracted SCL were separated by using one-dimensional TLC, utilizing benzene:hexane (1:1) as the first developing solvent for neutral lipids (squalene, cholesterol ester, waxes) (Fig 2), hexane:diethyl ether:glacial acetic acid (70:30:1) as the second

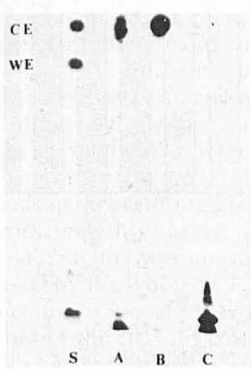

Figure 2. Thin-layer chromatographic plate sprayed with sulfonic acid. The SCL were separated with TLC using the first developing solvent (benzene:hexane, 1:1) for neutral lipids. *CE*, cholesterol esters; *WE*, wax ester; *S*, standard; *A*, stratum corneum lipid; *B*, cholesterol ester fraction; *C*, polar lipid fraction-I.

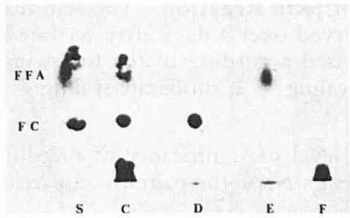

Figure 3. Thin-layer chromatographic plate sprayed with sulfonic acid. The polar lipid fraction-I obtained in Fig 2 was further separated with TLC using the second developing solvent (hexane:diethyl ether:glacial acetic acid, 70:30:1). *FFA*, free fatty acid; *FC*, free cholesterol; *S*, standard; *C*, polar lipid fraction-I; *D*, cholesterol fraction; *E*, free fatty acid fraction; *F*, polar lipid fraction-II.

for polar lipids (cholesterol, triglycerides, free fatty acids, and phospholipids) (Fig 3), and chloroform:methanol:distilled water (90:10:1) and petroleum ether:diethyl ether:glacial acetic acid (70:50:1) as the third for ceramide and glycolipids, respectively (Fig 4), as has been previously reported [7,8]. Individual bands of lipids were visualized after spraying sulfonic acid, identified by cochromatography against known standards, and then isolated by extracting with diethyl ether after scratching them. The isolated lipids, if necessary, were again applied on the same chromatographic system for further confirmation.

Application of Extracted Lipids Extracted lipids or their subfractions were solubilized at 10% concentration into squalane solution containing 1% α-monomethyl heptadecyl glyceryl ether (GE). This solution was applied daily at 0.014 ml/area (approximately 2 $\mu l/cm^2$) from the first day after 40-min acetone/ether treatment for 2 or 3 successive days.

Measurement of Water-Holding Capacity of the Stratum Corneum In Vivo Water-holding capacity of the stratum corneum was measured according to the method of Tagami et al. [9]. Changes in water-holding capacity of the treated areas were measured daily for 3 or 4 successive days by a capacitance conductance meter (model IB-354, IBS Inc., Japan). The treated areas were rinsed with water at 37°C and then after keeping volunteers at 20°C and 50% humidity for 20 min, were measured for skin reaction and conductance immediately before sample applications. Conductance measurements were carried out 5 times at the same area and the values were averaged to obtain individual values.

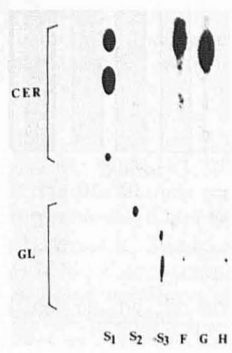

Figure 4. Thin-layer chromatographic plate sprayed with sulfonic acid. The polar lipid fraction-II obtained in Fig 3 was further separated with TLC using the third developing solvent (a; chloroform:methanol:distilled water, 90:10:1 and b; petroleum ether:diethyl ether:glacial acetic acid, 70:50:1) for ceramide and glycolipid, respectively. *CER*, ceramide; *GL*, glycolipid; *S1*, standard(ceramide); *S2*, standard(cerebroside); *S3*, combination of phosphatidyl ethanolamine and phosphatidyl cholyne; *F*, polar lipid fraction-II; *G*, ceramide fraction; *H*, glycolipid fraction.

Measurement of Skin Reaction The skin reaction, including scaling was observed over 4 days after acetone/ether treatment. Scaling was assessed according to the following scale: no scaling = 0, slight scaling = 1, moderate scaling = 2, marked scaling = 3.

Statistics The level of significance of the difference was calculated by Student's *t*-test for paired comparison or Cochran's Q-test.

RESULTS

Recovery by Crude Lipids An application of acetone/ether to human forearm skin for extremely prolonged periods of 5–20 min, as compared with the usual procedure for the extraction of skin surface lipids, induced an enduring (more than 4 days), chapped and scaly appearance of the stratum corneum without any inflammatory reaction [5]. Under these conditions, a significant decrease of conductance in the treated areas was observed when compared with the untreated control areas. This decreased conductance barely returned to the normal level by the fourth day after treatment.

Two daily topical applications of 10% SCL fraction in GE/squalane base on the lipid-depleted stratum corneum induced a significant recovery of the decreased conductance value as compared with nontreatment, or GE/squalane base only, whereas the SL did not show any significant recovery even in combination with GE/squalane (Fig 5). The recovery level by the SCL was significantly higher even when compared with 10% glycerine in the same GE/squalane system. Nevertheless, when GE was not added to the system, there was no significant recovery found with any of the lipid fractions. The observed recovery was specific for a combination with GE among the several surfactants used (data not shown).

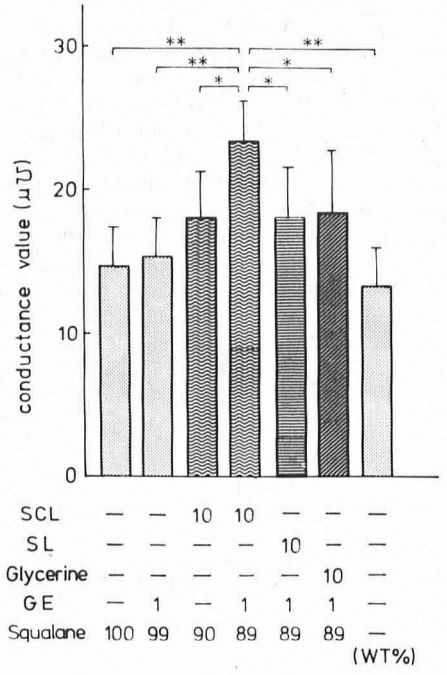

Figure 5. Recovery effect of extracted stratum corneum lipid (*SCL*) and sebaceous-rich lipids (*SL*) on the decreased conductance values induced by acetone/ether (1/1) treatment. The forearm skin of 10 healthy volunteers was treated with acetone/ether for 40 min (day −1). The extracted lipids (10%) were applied daily from day 0 to day 1. Conductance value was measured 2 days after the first application (day 0) in comparison with no applied area or GE/squalane base only. Each *bar* represents the mean conductance value of 10 volunteers ± SE. *GE*, α-monomethyl heptadecyl glyceryl ether; *, $p < 0.05$; **, $p < 0.01$.

Consistent with changes in the conductance value, the scaling which occurred after acetone/ether treatment had significantly decreased after the 2 daily applications with SCL as compared with no application, while SL and glycerin did not show any recovery in the same system (Fig 6).

Recovery by Separated Lipids Two daily topical applications of 5 separated lipid fractions (cholesterol ester, free fatty acid, cholesterol, ceramide, glycolipid) from the SCL at 10% concentration in the same system induced a significant increase in the conductance value as compared with GE/squalane base, especially when the ceramide fraction was used (Fig 7). Furthermore, the glycolipid and cholesterol fractions also exhibited a significant recovery as compared with no application. In contrast, free fatty acid and cholesterol ester fractions did not show any significant increase in their conductance value. In order to clarify the time course and dose dependency of the recovery in the conductance value, ceramide fractions at 3 and 10% concentrations in GE/squalane base, were applied daily for 3 successive days (from days 0 to 2) and their effect evaluated daily. A significant increase relative to the base solution was observed with 3 and 10% ceramide fractions by 2 days after the first application, with the 10% application showing a higher recovery than 3% (Fig 8).

DISCUSSION

Little is known about the function of SCL in the water-holding properties although available evidence [1,8] suggests a role for skin barrier function and stratum corneum cohesion. Our study has revealed that the SCL function as a water modulator in the intercellular spaces of the horny layers whose depletion and reinjection are primarily comparable with the loss and recovery of the water-holding properties. Because each lipid component and solubilized lipids in GE/squalane by themselves possess no substantial capacity for holding water, it is possible that applied lipids could be selectively compartmentalized into the intercellular spaces to exert the water-holding capacity. The importance of selectively entering the intercellular spaces and then being compartmentalized is corroborated by the fact that the addition of a specific

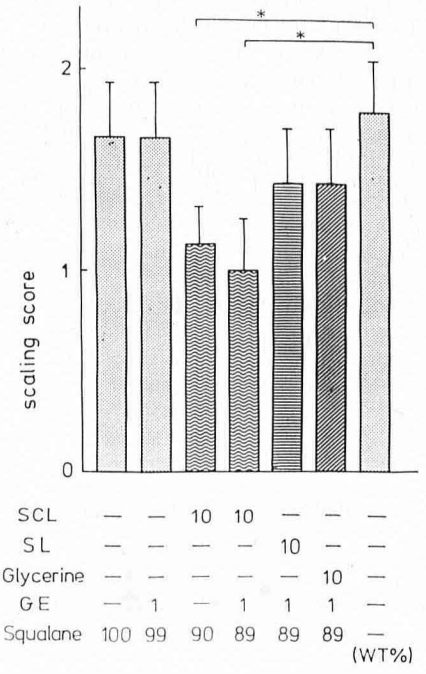

Figure 6. Recovery effect of extracted stratum corneum lipid (*SCL*) and sebaceous-rich lipids (*SL*) on induced scaling. Each *bar* represents the mean scaling score of 10 volunteers ± SE. *GE*, α-monomethyl heptadecyl glyceryl ether; *, $p < 0.05$.

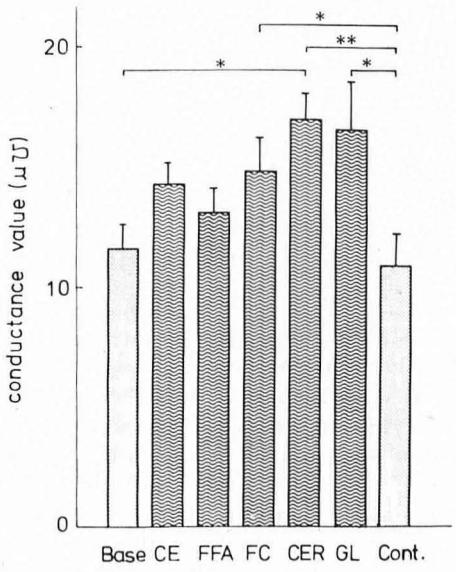

Figure 7. Recovery effect of chromatographically separated lipid components on the decreased conductance values induced by acetone/ether (1/1) treatment. The forearm skin of 10 healthy male volunteers was treated with acetone/ether for 40 min (day −1). The chromatographically separated lipids (10% in squalane containing 1% GE) were applied daily from day 0 to day 2. Conductance value was measured 2 days after the first application (day 0) in comparison with no applied area or base only. Each *bar* represents the mean conductance value of 10 volunteers ± SE. *Base,* 1% GE in squalane; *CE,* cholesterol ester fraction, *FFA,* free fatty acid fraction; *FC,* free cholesterol fraction; *CER,* ceramide fraction; *GL,* glycolipid fraction; *Cont.,* nontreated; *, $p < 0.05$; **, $p < 0.01$.

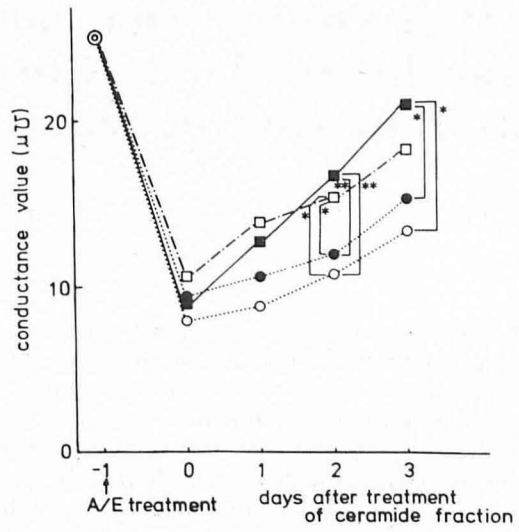

Figure 8. A time course and dose dependency of the recovery effect by ceramide fraction. The forearm skin of 10 healthy male volunteers was treated with acetone/ether (*A/E*) for 40 min (day −1). The chromatographically separated ceramide (10% in squalane containing 1% GE) was applied daily from day 0 to day 2. Conductance value was measured daily in comparison with no applied area or base only. ○, nontreated control; ●, base (squalane containing 1% GE); □, 3% ceramide fraction in base; ■, 10% ceramide fraction in base; *, $p < 0.05$; **, $p < 0.01$.

nonionic surfactant such as alkyl glyceryl ether which easily forms a lamellar structure in combination with lipids [10] is necessary for the insertion of the lipid components into the stratum corneum in order for it to improve its water-holding capacity.

Grayson and Elias [6] reported that the SCL are mainly derived from membrane complexes whose preparations account for approximately 80% of the total SCL. It has been well established [8] that the SCL consist of neutral (60–80%) and sphingolipids (15–35%). The sphingolipids comprise over 80% ceramides vs lesser quantities of glycosphingolipids. As the spingolipid content is reported to reveal a direct relationship with permeability to water, with the concept that the skin site which demonstrates the greatest permeability contains the greatest quantities of sphingolipids [8], it may be possible that the stratum corneum could exert the water-holding properties in a manner similar to the way it controls the water permeability which could be controlled to some extent by the ceramide content.

Although physical properties of the SCL, which are essential for holding water in the stratum corneum, are not yet known, it is of interest to note that the precise structure of the naturally occurring ceramides in the mammalian stratum corneum has been shown to contain many hydroxyl groups besides the amide group [11] whose existence in lipophilic compounds can interact with water by forming a network of hydrogen bonds [12]. An important observation made in the restoring process of the deranged water-holding properties was that the recovered level in conductance by the ceramide fraction was not necessarily equivalent to the corresponding level by the whole SCL fraction, suggesting the there is a definite contribution of other neutral lipids to the water-holding properties of the stratum corneum.

Since ceramides are shown to function as amphipathic materials to form lipid bilayers [12], our findings that the ceramide fraction exhibits the highest recovery potential for water-holding properties suggest the possibility that the hydrogen bonding groups in ceramides, when arranged in the broad lipid bilayers in combination with other neutral lipids, play a functional role for the water-holding properties of the stratum corneum. Experiments currently in progress on the interaction of naturally occurring ceramides with other lipids in the stratum corneum are necessary for clarifying the mechanism underlying the water-holding properties.

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