**Effect of topically applied sphingomyelin-based liposomes on the ceramide level in the three dimensional cultured human skin model**

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Abstract

Sphingomyelin-based liposomes were prepared and applied on the stratum corneum side or basal layer side of three-dimensional cultured human skin model, and the increase in type II ceramide (ceramide II) level was evaluated in the cultured skin model. The sphingomyelin-based liposomes were prepared by a high pressure emulsification method and the obtained liposomes were characterized; particle diameter and zeta potential of the liposomes were 155.3 nm and -11.4 mV, respectively. Spherical shape and lamella structure were observed by transmission electron microscopy. The sphingomyelin-based liposomes or saline were applied on the cultured skin model, and ceramide II was extracted from the skin model. The extracted ceramide II was separated by an HPTLC and quantified by a densitometer. The amount of ceramide II in the cultured skin model was significantly increased by application of the sphingomyelin-based liposomes compared with the non-application group. Thus, the sphingomyelin-based liposomes are useful to enrich the ceramide level in the three dimensional cultured skin model.

Keyword: sphingomyelin, liposome, ceramide, skin, three-dimensional cultured human skin model

1. Introduction

Transdermal drug delivery offers many potential advantages over conventional methods for drug delivery (Schreier, 1994, Cevc, 1996, Touitou, 2000, Barry, 2001). Skin has been used as an application site of therapeutic drugs to avoid the hepatic first pass effect and side effects in the GI tract. Thus, topical formulations applied on skin are beneficial, especially for infants and elder patients who sometimes have difficulty for swallowing medicines. Recently, several functional cosmetics were developed and used. These articles are very similar to topical drug formulations like creams, ointments, gels, lotions and etc. Some of active cosmetic ingredients must penetrate skin to have effective function for cosmetics.

However, the uppermost layer of the skin, the stratum corneum (SC), having 10-40 μm-thick except for the palm of hands and feet bottoms, and composing of partly flattened and keratinized layers, acts as an excellent intrinsic barrier for preventing water transpiration from inside of the body and against invasion of several chemicals and drugs (Abraham, 1990, Elias, 1975, Holleran, 1991). Thus, the SC plays an important role as a penetration barrier against most therapeutic drugs. SC has the special structure having keratinocytes as blicks and interceller lipids filled up as mortar structure (Elias, 1983, Uchida, 1988). Intercellular lipids of the SC were composed by sphingolipids like ceramides, cholesterols, cholesterol esters and fatty acids (Swartzendruber, 1989, Bowstra, 1995). Particularly, ceramides which are half of the intercellular lipids, have the most important roles for the barrier function in SC (Imokawa, 1985, Imokawa, 1991). Disruption or lack of the SC lipids decreased the skin barrier function and skin-moisturising function. They caused dry skin and sometimes several skin diseases. It is also reported that sphingolipids like ceramides significantly decreased and inhibited the barrier function of skin especially in atopic dermatitis-patients

(Imokawa, 1991, Jensen, 2004; Farwanah, 2005).

Major ceramides in the SC are derived from glucosylceramide and sphingomyelin (Fig. 1). All species of ceramides are available through glucocerebrosidase activity, while only two species (type II and V ceramide) are generated through hydrolysis of sphingomyelin by sphingomyelinase (Motta, 1995, Jensen, 2004, Vicanova, 1998), as explained in Fig. 1. These ceramides are then metabolized to sphingosine and free fatty acids by ceramidase (Yada, 1995). Thus, the amount of ceramides in SC is controlled by the three enzymes; glucocerebrosidase, sphingomyelinase and ceramidase.

Figure 1

Sphingomyelin is one of the phospholipids which are primary components of biomembrane. It is hydrolyzed by sphingomyelinase in process of the differentiation from granular cells to corneocytes in the epidermis, and metabolized to ceramides and phosphocholine (Grayson, 1985) (Fig. 1). Therefore, ceramide level in skin may be increased, if sphingomyelin is successfully delivered to the viable epidermis. However, sphingomyelin is difficult to formulate in the topical formulations and cosmetics, since this phospholipid is not soluble and stable in the aqueous formulations which are frequently used for cosmetics.

In this paper, sphingomyelin-based liposomes without entrapped drugs and active ingredients were prepared and utilized to enrich ceramides in skin, since liposomes are one of the useful formulations for phospholipids as well as entrapped novel drugs. The latter is broadly used to deliver doxorubicin, amphotericin B, and so on in patients. The presently prepared liposomes were characterized by their particle diameter, zeta potential and TEM

images. Then, the sphingomyelin-based liposomes were applied to three-dimensional cultured human skin model to evaluate whether they can increase ceramide level in the cultured skin.

## 2. Materials and Methods

### 2-1. Materials

Sphingomyelin from milk was purchased by NOF Corporation (Tokyo, Japan). Ceramide II standard was obtained Nikko Chemical Co., Ltd. (Tokyo, Japan). [N-methyl-$^{14}$C] Sphingomyelin was purchased by GE Healthcare UK Ltd. (Buckinghamshire, U. K.). LabCyte Epi-Model (a three-dimensional cultured human skin model) was obtained Japan Tissue Engineering Co., Ltd. (Gamagori, Aichi, Japan). Sphingomyelin-based liposomes were prepared using a high pressure extrusion method by Daiichi Fine Chemical Co., Ltd, Takaoka, Toyama, Japan. In briefly, sphingomyelin was dissolved in ethanol. Distilled water and methylparaben were added and emulsified. It was extruded through the membrane filters (200nm) by high pressure. The liposomes are referred to PANASOME® SPM. Other reagents were obtained commercially and used without further purification

### 2-2. Characterization of liposomes

Particle diameter of sphingomyelin-based liposomes was measured by dynamic light scattering using a Zetasizer (3000HSA, Sysmex, Kobe, Japan). Structure of these liposomes was analyzed by negative stain with a transmission electron microscopy (JEM 1010, Jeol, Tokyo, Japan).

### 2-3. Measurement of ceramide amount in three dimensional cultured human skin model.

Three wells of LabCyte Epi-Model were washed by 1.0 mL of PBS and homogenized by 1.6 mL of 0.25 M sucrose. Separately, 0.24 μCi/mL of [N-methyl-$^{14}$C] Sphingomyelin (50 μL) was added in 20 μL of 1% Triton X-100 in acetate buffer (pH 5.0). Then, 30 μL of distilled water and 50 μL of 0.2 M acetate buffer (pH 5.0) were added and sonicated for 10 min. Then, diluted [N-methyl-$^{14}$C] sphingomyelin solution (50 μL) was added to 1.0 mL of the homogenized solution of cultured human skin samples. The sample (0.2 mL) was incubated at 37°C for 1, 2 and 3 hours. Chloroform: methanol (2: 1 v/v) (0.8 mL) was added to stop the reaction of sphingomyelin to phosphocholine and ceramides. The produced free [$^{14}$C]-phosphocholine was measured by a liquid scintillation counter (LSC-6100, Aloka, Tokyo, Japan).

2-4. Application of sphingomyelin-based liposomes and extraction of ceramide II from LabCyte Epi-Model.

1.0% Sphingomyelin-based liposomes in physiological saline or the saline alone (control) (1.0 mL each) were applied on the basal layer side or stratum corneum side of LabCyte Epi-Model for 24 hours. Sphingomyelin powder in physiological saline was also applied for comparison. Medium containing ascorbic acid was exchanged for enhancing keratinization. After 24 hours, ceramides in the cultured skin model were extracted by 6.0 mL of chloroform:methanol (2:1 v/v).

2-5. Lipid analysis by HPTLC (high performance thin-layer chromatography)

The extracted ceramides were separated by an HPTLC plate (Silica Gel 60, Merck, Darmstadt, Germany). The HPTLC was developed twice with chloroform:methanol:acetic acid = 190:9:1 (v/v). Ceramides were visualized by treatment with 10 % $CuSO_4$, 8 % $H_3PO_4$

aqueous solution and heating to 180 °C for 10 min. Ceramide II on the HPTLC plate was quantitatively determined by a densitometer.

2-6. Statistic analysis

The obtained data were represented as the mean ± S.D. of four experiments. The statistical test was done using Welch's t-test.

3. Results

3-1. Characterization of sphingomyelin-based liposomes

Sphingomyelin-based liposomes prepared by a high pressure emulsification method have no entrapped drugs or active ingredients for drug or cosmetic formulations. The liposomes were very stable even at 40 degree Celsius for 6 months. Particle size and zeta potential of sphingomyelin-based liposomes were measured by a dynamic laser scattering and an electrophoresis, respectively. The observed particle diameter was 155.3 ± 0.46 nm and zeta potential was and -11.4 mV. Figure 2 shows a transmission electron microphotograph of sphingomyelin-based liposome (Fig. 2a), suggesting that the sphingomyelin liposomes had a core shell and lamella structure, as like in general liposomes prepared by phosphatidylcholine and cholesterol (Fig 2b).

Figure 2a and 2b

3-2. Increase in ceramide II level in LabCyte Epi-Model

Figure 3 shows the application effect of sphingomyelin-based liposomes on the increased amount of ceramide II in LabCyte Epi-Model. [$^{14}$C]sphingomyelin was applied

to LabCyte Epi-Model, and the amount of the [$^{14}$C] phosphocholine was measured as an index of generation of ceramide II. Sphingomyelinase activity was measured as the amount at 1, 2 and 3 hours after application of sphingomyelin-based liposomes. The amount of ceramide II was 2.1, 3.9 and 7.8 nmol/well, respectively. The amount of ceramide II in the LabCyte Epi-Model was linearly increased to 3 hours.

Figure 3

3-3. Change of ceramide II amount in LabCyte Epi-Model after application of sphingomyelin-based liposomes on the basal layer side of the cultured skin model.

Sphingomyelin-based liposomes were applied to the basal layer side of epidermis of LabCyte Epi-Model for 6 hours. The skin model was cultured in an enhancing keratinized medium containing ascorbic acid for 12 or 24 hours. Ceramide II was separated from the cultured skin model and determined by an HPTLC. Figure 4a (12 h cultivation) and b (24 h cultivation) show the amount of ceramide II in the cultured skin model. The ceramide II amount in the skin model was significantly increased after application of sphingomyelin-based liposomes, being about 1.82 and 2.04 -fold higher for 12 and 24 hour cultivation, respectively, than that without liposome-application.

Figure 4a and b

3-4. Change in ceramide II amount in LabCyte Epi-Model after application of sphingomyelin-based liposomes on the SC side.

Next, sphingomyelin-based liposomes were applied to the SC side for 24 or 48 hours.

The same cultivation was done as above. Ceramide II was separated from the cultured skin model and determined by an HPTLC. Figure 5a (24 hr application) and b (48 hr application) show the amount of ceramide II in the skin model. The ceramide II level was also significantly increased after application of the liposomes on the SC side, being about 0.90 and 1.24-fold higher with 24 and 48 hour cultivation, respectively, than that without liposome application.

Figure 5a and b

**4. Discussion**

Liposomes were prepared using sphingomyelin mostly and applied on the basal layer or SC side of a three-dimensional cultured human skin model, LabCyte Epi-Model, to investigate change in the ceramide II level in the cultured skin model. Chemical structure of the ceramides was identified as seven kinds by constitution of free fatty acids and hydroxyl-groups. In the present manuscript, production of type II ceramide was focused, because this type of ceramide is mostly effective against the skin moisturization in human face and body. The sphingomyelin-based liposomes prepared by a high pressure emulsification were characterized: particle diameter and zeta potential of the liposomes were 155.3 nm and -11.4 mV, respectively, and they have lamella and spherical structure, which was also evaluated by transmission electron microscopy (Fig. 2). The sphingomyelin-based liposomes were applied to LabCyte Epi-Model to examine the sphingomyelinase activity or ceramide level. This is the first report showing the sphingomyelinase activity in the three-dimensional cultured human skin model. The ceramide II amount was linearly increased for 3 hours after application of sphingomyelin-based liposomes (Fig. 3). These

results suggest that the cultured skin model has sphingomyelinase activity. When sphingomyelin-based liposomes were applied to LabCyte Epi-Model, ceramide II level was started to increase compared to that without the liposome application (Figs. 4 and 5). Ceramides were generated from sphingomyelin as a formulation of liposomes in LabCyte Epi-Model. Real human skin also has sphingomyelinase activity. Change in ceramide II amount in LabCyte Epi-Model after application of sphingomyelin powder in physiological saline as well as the sphingomyelin-based liposomes on the SC and basal layer side (data not shown). Ceramide II amount in the skin model by the SC-side application was similar to that by basal layer-side application. However, sphingomyelin-based liposomes have a great advantage compared with sphingomyelin powder in saline from view points formulation stability and easy to preparation.

Thus, the present sphingomyelin-based liposomes must be effective to enrich the ceramide level in human skin. In addition, liposome formulation is very useful to apply sphingomyelin on skin, since the lipid itself is difficult to formulate in conventional formulations. Type II and V ceramides are reported to be produced from sphingomyelin in the viable epidermis by sphingomyelinase (Jensen, 2004). In a future study, it is necessary to investigate the change in the amount of other ceramides except type II ceramide.

## Conclusion

Sphingomyelin-based liposomes were applied on the basal layer or SC of three-dimensional cultured human skin model, and the ceramide II level was increased by the liposomes. These results suggest that the sphingomyelin-based liposomes may have a moisturizing effect and barrier-keeping function in SC.

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Figure Captions

Fig. 1 Metabolic pathway of ceramides in epidermis

Fig. 2 TEM image of sphingomyelin-based liposome (a) and phosphatidylcholine / cholesterol = 7/3 (m/m%) liposomes (b)

Fig. 3 Increase in ceramide II amount in LabCyte Epi-Model

Fig. 4 Change of ceramide II amount in LabCyte Epi-Model after application of sphingomyelin-based liposomes on the basal layer side of the cultured skin model. Sphingomyelin liposomes were applied to the basal layer side of epidermis for 6 hours. After the skin model was cultured in an enhancing keratinized medium containing ascorbic acid for 12 (a) or 24 (b) hours. The data represent the average and standard deviation (n=4). * and ** : $p < 0.05$ and $p < 0.01$, respectively, compared to the control group. SPM-L: sphingomyelin-based liposomes.

Fig. 5 Change in ceramide II amount in LabCyte Epi-Model after application of sphingomyelin-based liposomes on the stratum corneum side of the cultured skin model. Sphingomyelin liposomes were applied to the stratum corneum side for 24 (a) or 48 (b) hours, after the skin model was cultured in an enhancing keratinized medium containing ascorbic acid for 24 hours. The data represent the average and standard deviation (n=4). ** : $p < 0.01$, N.S.: not significant, respectively, compared to the control group. SPM-L: the same as in Fig. 4.