Application of glucosylceramide based liposomes increased the ceramide content in a three-dimensional cultured skin epidermis

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Abstract

Ceramide is an intercellular lipid of the stratum corneum and is one of the most important components of the epidermal permeability barrier. Glucosylceramide, a ceramide precursor, was applied to three-dimensional skin culture to regulate ceramide. Glucosylceramide (GlcCer)/ dimyristoyl phosphatidylcholine (DMPC)= 4/ 4 (m/ m%), GlcCer/ DMPC/ dimyristoyl phosphatidylglycerol (DMPG)= 4/ 4/ 1(m/ m%) liposomes were prepared by the thin layer method. The particle diameters of GlCCer/ DMPC and GlcCer/ DMPC/ DMPG liposomes were 124.0±0.6 and 119.3±18.9 nm, and the zeta potentials were 1.3±0.3 and -19.9±0.3 mV, respectively. Stability of these GlcCer liposomes was measured by transmission light scattering. Transmission light scattering of neutral charged GlcCer (GlcCer/DMPC) liposomes increased in a time dependent manner. In contrast, negatively charged GlcCer (GlcCer/DMPC/DMPG) liposomes were not changed. β-glucocerebrosidase activity was measured in a cultured human skin model. Results confirmed that the cultured human skin model has β-glucocerebrosidase activity. GlcCer/DMPC/DMPG liposomes were applied to the three dimensional cultured human skin model, and ceramide NS, NP, AS, and AP were extracted from it. The various extracted ceramides were separated by HPTLC and quantified by a densitometer. The amount of ceramide AS only in the cultured skin model was significantly higher with the application of glucosylceramide-based liposomes than that of the non-application group, and was also dose dependent. Thus, glucosylceramide-based liposomes are useful for enriching the ceramide AS levels in a three dimensional cultured skin model.

Keywords: stratum corneum, ceramide, glucosylceramide, liposome, three dimensional cultured skin

Introduction

The uppermost layer of the skin, the stratum corneum (SC), which is 10-40 µm-thick, except for the palm of the hands and the soles of the feet, is composed of partly flattened and keratinized layers and acts as an excellent intrinsic barrier for preventing water transpiration from the body and the invasion of several chemicals and drugs [1- 3]. The SC has a special structure with keratinocytes acting as bricks and intercellular lipids acting as a mortar-like structure [4, 5]. The intercellular lipids of the stratum corneum include ceramides, cholesterol, cholesterol esters, and fatty acids [6, 7]. In particular, ceramides, the primary constituents of intercellular lipids, play the most important roles in the barrier function of the SC [8, 9]. The characteristic structure of ceramide is a free fatty acid and sphingosine or sphingolipid. The major ceramide of human stratum corneum has been classified into more than nine species by recent analysis techniques [10]. Disruption or a lack of SC lipids decreases skin barrier function and skin-moisturization, causing dry skin and sometimes several skin diseases. It was also reported that sphingolipids significantly decreased and inhibited the barrier function of the skin, especially in atopic dermatitis-patients [11, 12]. Therefore, the ceramide content in the skin may be increased if glucosylceramide is successfully delivered to a viable epidermis. However, ceramide is difficult to formulate in topical formulations and cosmetics since it is not soluble or stable in aqueous formulations, which are frequently used for cosmetics.

Serine palmitoyl-CoA transferase (SPT) is the key enzyme in skin sphingolipid biosynthesis. It is generated through two biosynthesis pathways, glucosylceramide hydrolysis through glucocerebrosidase [13, 14] and sphingomyelin hydrolysis through sphingomyelinase [15]. Therefore, if glucosylceramide is hydrolyzed by β -glucocerebrosidase, the ceramide content in the skin will increase.

We reported that application of sphingomyelin-based liposomes (SPM-L) to a three- dimensional cultured human skin model increased its type II ceramide (ceramide NS) content [16]. In particular, the small size of SPM-L is most effective at increasing ceramide levels [17].

In this article, glucosylceramide based liposomes were prepared and characterized. These

glucosylceramide based liposomes were applied to a three dimensional cultured human skin model.

Materials and Methods

Materials

Glucosylceramide (GlcCer) from rice bran was kindly provided by Okayasu Shoten Co. Ltd. (Saitama, Japan). Dimirystoyl phosphatidyl choline (DMPC) and dimirystoyl phosphatidyl glycerol (DMPG) were provided by Nippon Fine Chemical Co. Ltd. (Hyogo, Japan). Hydroxy and non-hydroxy ceramide standards were purchased from Matreya, LLC (Pleasant Gap, PA, USA). Ceramide AS and AP were obtained from Evonik Goldschmidt GmbH (Goldschmidtstrasse, Essen, Germany). Conduritol B epoxide (CBE) was perchased from Santa Cruz Biotechnology Inc. (Delaware, CA, USA). LabCyte Epi-Model (a three-dimensional cultured human skin model, 0.86 cm^2/well), medium and enhanced keratinization medium were obtained from Japan Tissue Engineering Co., Ltd. (Gamagori, Aichi, Japan). Phosphate-buffered saline (PBS) powder was obtained from Sigma (St. Louis, MO, USA). Silica gel 60 (Merck, Darmstadt, Germany) was used for the HPTLC plate. All other reagents were obtained commercially and used without further purification.

Preparation of glucosylceramide based liposomes (GlcCer-L)

For the assay of this experiment, liposomes composed of GlcCer and DMPC= 4/4 or GlcCer, DMPC and DMPG= 4/ 4/ 1 (as a molar ratio) and 0, 5, 10 and 20 mg/mL of final concentration of GlcCer were used and control liposome was prepared without GlcCer. At first, these components were dissolved in chloroform/methanol. The solvent was evaporated and dried using a rotary evaporator under 40 °C and 50 hPa before being stored in a vacuum for at least 1 h. The resulting thin lipid film was hydrated with PBS (pH 7.4). Liposomes were then subjected to freezing and thawing for five cycles with liquid nitrogen and extruded five times through polycarbonate double-membrane filters using an extruder (pore-size: 100 nm; Nucleopore, Costar, Cambridge, MA, USA). Finally, the GlcCer-L suspension was separated by ultracentrifugation at 250,000×g for 15

min (Himac CS100 GXL, Hitachi, Tokyo, Japan). The resultant liposomal pellets were resuspended in PBS (pH 7.4).

Characterization of liposomes

The size distribution of GlcCer-L was measured by dynamic light scattering using a Zetasizer (3000HSA, Sysmex, Kobe, Japan). The structure of these liposomes was analyzed by a negative stain with transmission electron microscopy (JEM 1010, Jeol, Tokyo, Japan).

Measurement of glucocerebrosidase in a three dimensional cultured human skin model.

12 wells of LabCyte Epi-Model was minced with a scissors on ice. Skin samples were homogenated using Teflon homogenizer (1,000 rpm, 3 strokes) in 0.25 M sucrose solution. The homogenate was centrifuged under conditions of 600 ×g, 10 minutes and supernatant was assumed skin sample. Separately, 2.5 μM of C12-NBD-GlcCer was added to 20 μL of 0.6% sodium taurocholate and 0.25 % Triton X-100 in 50 mM phosphate-citrate buffer (pH 5.0). Diluted C12-NBD-GlcCer solution (20 μL) was added to 180 μL of the homogenized solution (0.05 %) of cultured human skin samples or hairless mice skins. The sample (0.2 mL) was incubated at 37°C for 15, 30, 45, and 60 min. The reaction was stopped by adding 200 μL chloroform: methanol (2: 1 v/v). The produced C12-NBD-Cer was measured by high performance liquid chromatography [18].

Application of glucosylceramide-based liposomes and extraction of ceramide from the LabCyte Epi-Model.

5, 10, and 20 mg/mL of GlcCer-L in physiological saline, DMPC/DMPG liposome or saline alone (control) (0.3 mL each) was applied to the stratum corneum side of the LabCyte Epi-Model once per day for 7 days. Cultures of the LabCyte Epi-model were grown at 5 % CO_2 and 37°C. The medium (1.0 mL) was changed every day. After 7 days, 1.0 mL of the culture medium containing ascorbic acid was then exchanged for enhanced keratinization medium in 24 hours, the ceramide molecules were extracted

from the cultured skin model using 6.0 mL of chloroform: methanol (2:1 v/v). was cocultured The β-glucocerebrosidase inhibition experiment co-cultured 1 % or 3 % Conduritol B epoxide (CBE) and 10 mg/mL of glucosylceramide liposome (GlcCer/DMPC/DMPG= 4/ 4/ 1) for seven days.

The extraction method and lipid analysis by HPTLC (high performance thin-layer chromatography)

Various ceramides in the LabCyte Epi-Model was extracted using the Bligh and Dyer method [19]. Briefly, the Labcyte Epi-model was dissolved in 6 mL of chloroform/methanol (2/1 v/v) and sonicated (70 W, 10 min) by a probe type sonicator (Sonifire B-12, Branson Ultrasonics, CT, USA). The ceramide extracted solution was dried by nitrogen gas, and resolved 0.4 mL of chloroform/methanol (2/1 v/v). Various ceramide extracts were separated using an HPTLC plate (Silica Gel 60, Merck, Darmstadt, Germany). HPTLC was developed twice with chloroform: methanol: acetic acid = 190: 9: 1 (v/v). Ceramide molecules were visualized by treatment with 10 % $CuSO_4$, 8 % H_3PO_4 aqueous solution, and heating to 180 °C for 10 min. The amounts of the various types of ceramide (Ceramide NS, NP, AS, and AP) on the HPTLC plate were quantitatively determined using a densitometer.

Statistical analysis

The obtained data were represented as the mean ± S.D. of four experiments. Statistical tests was performed using Dunnet’s multiple comparison test (SAS ver. 9.2).

Results

Characterization of glucosylceramide-based liposomes

Glucosylceramide-based liposomes prepared by a hydration method and freeze-thawing method have no entrapped drugs or active ingredients for drug or cosmetic formulations. The particle diameter and zeta potential of sphingomyelin-based liposomes were measured by dynamic laser scattering and electrophoresis, respectively. Liposomes were not prepared only by glucosylceramide. Therefore, GlcCer/ dimyristoyl phosphatidylcholine (DMPC)= 4/ 4 (m/ m%, total lipid concentration: 26 μmol), GlcCer/ DMPC/ dimyristoyl phosphatidylglycerol (DMPG)= 4/ 4/ 1 (m/ m%, total lipid concentration: 29.25 μmol) was prepared by the thin layer method. And DMPC/DMPG= 4/1 control liposomes (without GlcCer, total lipid concentration 16.25μmol) were prepared by same method. GlcCer The particle size and zeta potential of the liposomes was summarized in Table 1. The particle diameters of GlCCer/ DMPC= 4/4, GlcCer/ DMPC/ DMPG= 4/ 4/ 1 and DMPC/DMPG=4/1 were 115, 120 and 137 nm and the zeta potentials were 5, -20 and -36.1 mV, respectively. Figure 1 shows transmission light scattering was used to measure liposomes by Turbiscan® MA2000 (Formulaction, FL, USA). Transmission light scattering of neutral charged GlcCer liposomes (GlcCer/DMPC) increased in a time dependent manner. On the other hand, negatively charged GlcCer liposomes (GlcCer/DMPC/DMPG) were not changed. From these results, subsequent examinations used GlcCer/ DMPC/ DMPG. Figure 2 shows a transmission electron microphotograph of GlcCer/ DMPC/ DMPG=4/ 4/ 1 (Fig. 2a), suggesting that glucosylceramide-based liposomes had a core shell and lamella structure, similar to general liposomes prepared by phosphatidylcholine and cholesterol (Fig 2b).

Figure 1

Figure 2a and 2b

Glucocerebrosidase activity in the LabCyte EPI-MODEL

Figure 3 shows glucocerebrosidase activity in the LabCyte EPI-MODEL. Glucocerebrosidase activity was assessed by measuring NBD-ceramide. Glucocerebrosidase activity was measured at 15, 30, 40, and 60 minutes, respectively. The amount of ceramide was 15.0, 28.0, 42.8, and 49.7 μmol/mg protein after application of NBD- glucosylceramide.

Figure 3

Changes in ceramide amounts in the LabCyte EPI-MODEL after application of glucosylceramide liposomes.

0 (DMPC/DMPG liposomes), 5, 10, and 20 mg/mL of glucosylceramide liposomes were applied to the stratum corneum side for 7 days. The skin model was then cultured in enhanced keratinization medium containing ascorbic acid for 24 hours before the ceramides were separated from the cultured skin model and determined by HPTLC. Figures 4a (ceramide NS), 4b (ceramide NP), 4c (ceramide AS), and 4d (ceramide AP) show the amounts of each ceramide in the cultured skin model. The amounts of ceramide AS were increased by application of glucosylceramide liposomes in a dose dependent manner. However, ceramide NS, NP, and AP were not changed by glucosylceramide liposome application. All ceramides were not changed by DMPC/DMPG control liposomes (without GlcCer) application. Figure 5 shows HPTLC images.

Figure 4a, 4b, 4c and 4d

Figure 5

Effect of the inhibitor for β-glucocerebrosidase on ceramide amount in the LabCyte EPI-MODEL after application of glucosylceramide liposomes.

1 % or 3 % CBE and 10 mg/mL of glucosylceramide liposomes were co-cultured to the stratum corneum side for 7 days. The skin model was then cultured in enhanced keratinization medium containing ascorbic acid for 24 hours before the ceramides were separated from the cultured skin model and determined by HPTLC. Figures 6a (ceramide NS), 6b (ceramide NP), 6c (ceramide AS), and 6d (ceramide AP) show the amounts of each ceramide in the cultured skin model. The amounts of ceramide NS, NP, AS and AS were significantly decreased by application of β-glucocerebrosidase inhibitor.

Figure 6a, 6b, 6c and 6d

Discussion

Major lipid components in the stratum corneum, ceramide species, were generated through glucosylceramide hydrolysis by glucocerebrosidase in the epidermis [13, 14]. Ceramide is a major component in the stratum corneum. It is reported that the stratum corneum ceramide content of atopic dermatitis's patients was decreased [11, 12]. It is very important to increase ceramide levels in the stratum corneum of atopic dermatitis patients effectively to maintain skin barrier function and the water content of the skin. We reported that stratum corneum ceramides were increased by applying sphingomyelin liposomes to three-dimensional cultured skin [16, 17]. In this article, glucosylceramide based liposomes were prepared and applied on the three dimensional cultured human skin model.

Liposomes are a vehicle composed of phospholipids for the administration of pharmaceutical drugs and cosmetic formulations [20- 22]. At first, liposomes were not able to be prepared in glucosylceramide alone. It was assumed that glucosylceramide had extremely high lipid solubility. Therefore, liposomes were prepared and added in a same molar of glucosylceramide and dimyristoyl phosphatidyl choline (GlcCer/DMPC liposomes). As a result, GlcCer/DMPC liposomes were able to be prepared. Changes in transmission light scattering of these liposomes were observed by Turbiscan® MA2000. Transmission light scattering was greatly affected, and it was suggested that the stability of the GlcCer/DMPC liposomes was unstable. As the surface charge of the GlcCer/DMPC liposomes we used in this study is neutral, its liposomes aggregated with each other to form secondary particles. Furthermore, dimyristoyl phosphatidyl glycerol was added to add a surface charge to the liposomes (GlcCer/DMPC/DMPG). Transmission light scattering changes in GlcCer/DMPC/DMPG liposomes were not observed. GlcCer/DMPC/DMPG liposomal surfaces have a negative charge and it was electronically repelled and was stable. It was decided to perform a future study using stable GlcCer/DMPC/DMPG liposomes. The internal structure of glucosylceramide based liposomes was observed using transmission electron microscopy. It was revealed to be a vesicle having a lamellar structure. Its glucocerebrosidase activity was examined in a three dimensional human cultured epidermis model. NBD-labeled

glucosylceramide was used as a substrate. Results show that glucosylceramide was produced in a time dependent manner. In addition, a similar result was obtained using in mice skin (data not shown). It was revealed that the culture epidermis model had glucocerebrosidase activity. The possibility that ceramide was produced by glucocerebrosidase in the epidermis if GlcCer was delivered in the culture epidermis was shown.

Ceramide NS, NP, AS, and AP were quantitated after application of 5, 10, and 20 mg/mL of glucosylceramide liposomes. The amount of ceramide AS increased with application of glucosylceramide liposomes in a dose dependent manner. However, ceramide NS, NP, and AP were not changed by glucosylceramide liposome application. And, the content of ceramides significantly decreased by an application of the inhibitor for ß-glucocerebrosidase (conduritol B epoxide). The glucosylceramide, in which ceramide AS connected to glucose, was used in this study. It was suggested that the glucosylceramide applied to the skin epidermis was metabolized by glucocerebrosidase resulting in an increase in ceramide AS.

Generally, it was reported that all ceramide, except acylceramide (ceramide EOS, EOH, EOP), increases from glucosylceramide in the epidermis. When glucosylceramide is applied to the skin epidermis, various enzyme expression levels of ceramide in the skin epidermis, except ceramide AS, may increase.

In a future study, it is necessary to confirm changes in the amounts of ceramides, except ceramide AS. Thus, the present sphingomyelin-based liposomes were effective at enriching the ceramide levels in human skin. In addition, liposome formulations are very useful for the application of glucosylceramide to the skin since the lipid itself is difficult to formulate in conventional formulations. In a future study, it is necessary to investigate changes in the amounts of other ceramides.

Conclusion

Glucosylceramide-based liposomes were applied to the SC of a three-dimensional cultured human skin model, and ceramide AS levels increased. These results suggest that glucosylceramide-based liposomes have a moisturizing effect and a barrier-maintaining function in the SC.

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Figure Legends

Fig. 1 Transmission profile of GlcCer/DMPC/DMPG= 4/4/1 (m/m %, A) and GlcCer/DMPC= 4/4 (m/m %, B)

Fig. 2 Transmission electron microscopy images of GlcCer/DMPC/DMPG (A) and phosphatidylcholine/ cholesterol liposomes (B).

Fig. 3 Linear increase in β-glucocerebrosidase activity with the passage of time in the LabCyte EPI-MODEL

Fig. 4 Changes in the ceramide content of the LabCyte Epi-Model after application of glucosylceramide-based liposomes (GlcCer/DMPC/DMPG) to the skin model. (a) Ceramide AS, (b) Ceramide AP, (c) Ceramide NS and (d) Ceramide NP. 0, 5, 10, and 20 mg/mL of glucosylceramide-based liposomes were applied to the stratum corneum side of the epidermis for 7 days after it had been cultured in an enhanced keratinization medium containing ascorbic acid for 24 hours. Each data point represents the mean and standard deviation of at least three independent experiments. **, and ***: $p < 0.01$, and $p< 0.001$, respectively, compared to the control group.

Fig. 5 HPTLC image of a LabCyte Epi-model after application of glucosylceramide-based liposomes. Lane A: control (PBS), Lanes B, C and D: 5, 10, and 20 mg/mL of glucosylceramide-based liposomes, respectively.

Fig. 6 Effect of the inhibitor for β-glucocerebrosidase on ceramide amount in the LabCyte EPI-MODEL after application of glucosylceramide-based liposomes. (a) Ceramide AS, (b) Ceramide AP, (c) Ceramide

NS and (d) Ceramide NP. 1 % or 3 % CBE and 10 mg/mL of glucosylceramide liposomes were co-cultured to the stratum corneum side for 7 days after it had been cultured in an enhanced keratinization medium containing ascorbic acid for 24 hours. Each data point represents the mean and standard deviation of at least three independent experiments. **: $p < 0.01$, compared to the glucosylceramide liposomes group.

Table 1 Composition of GlcCer based liposomes and control liposomes

	Composition (molar ratio)	Particle diameter (nm)	Zeta potential (mV)
GlcCer	100%	-	-
GlcCer/DMPC	4/4	124.0±0.6	1.3±0.3
GlcCer/DMPC/DMPG	4/4/1	119.3±18.9	-19.9±0.3
DMPC/DMPG (control liposome)	4/1	137.0±0.4	-36.1±0.9

-: Liposomes were not prepared in this composition

Data represents mean±S.D. of four experiments

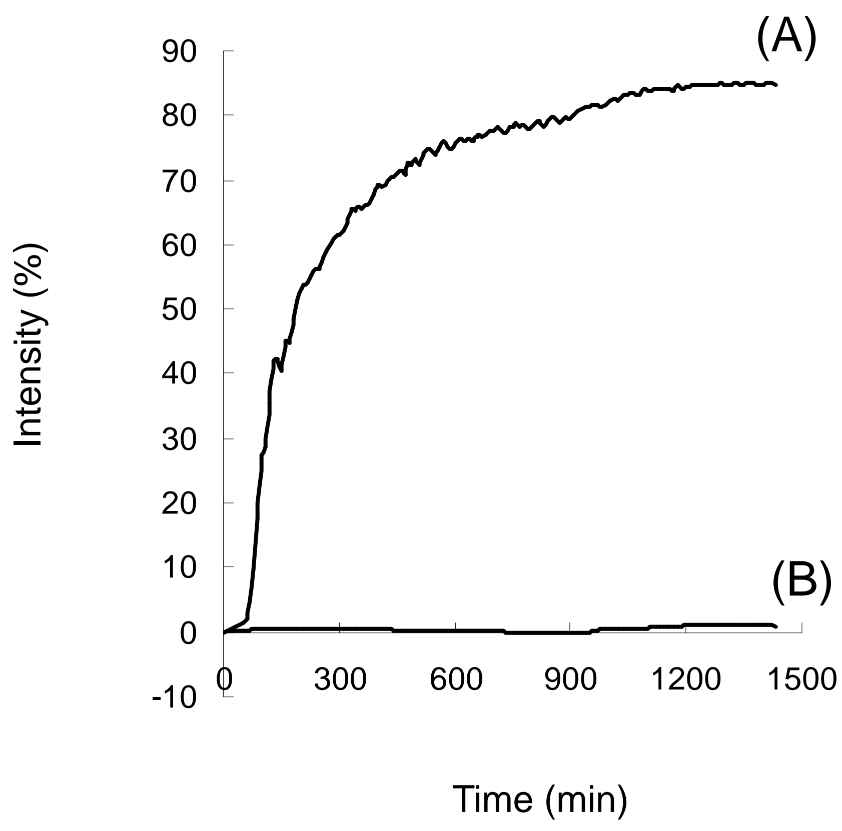
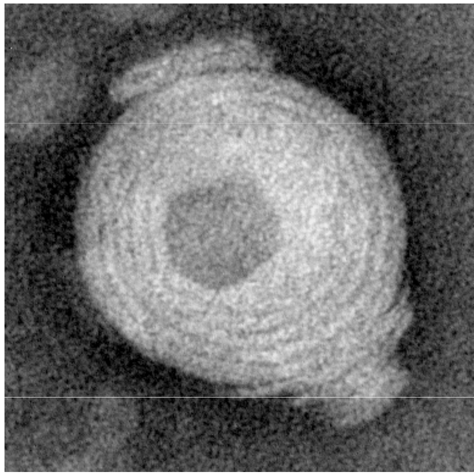


Figure 1

(A)



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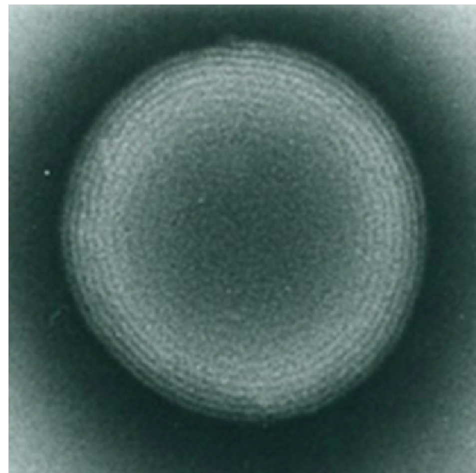
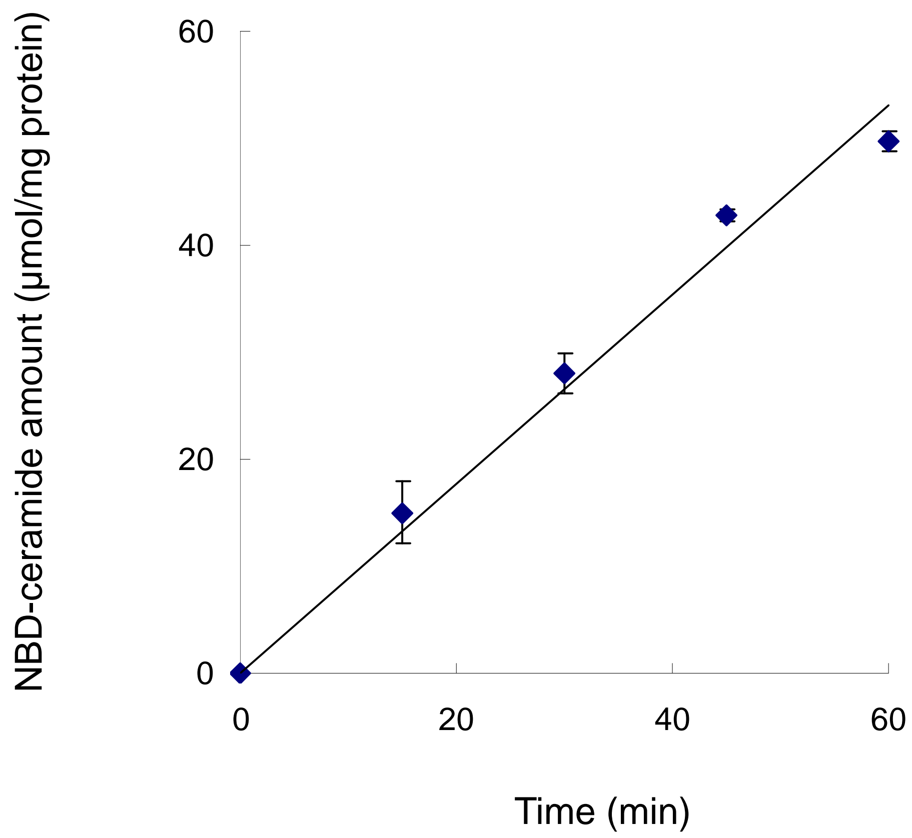


100 nm

Figure 2



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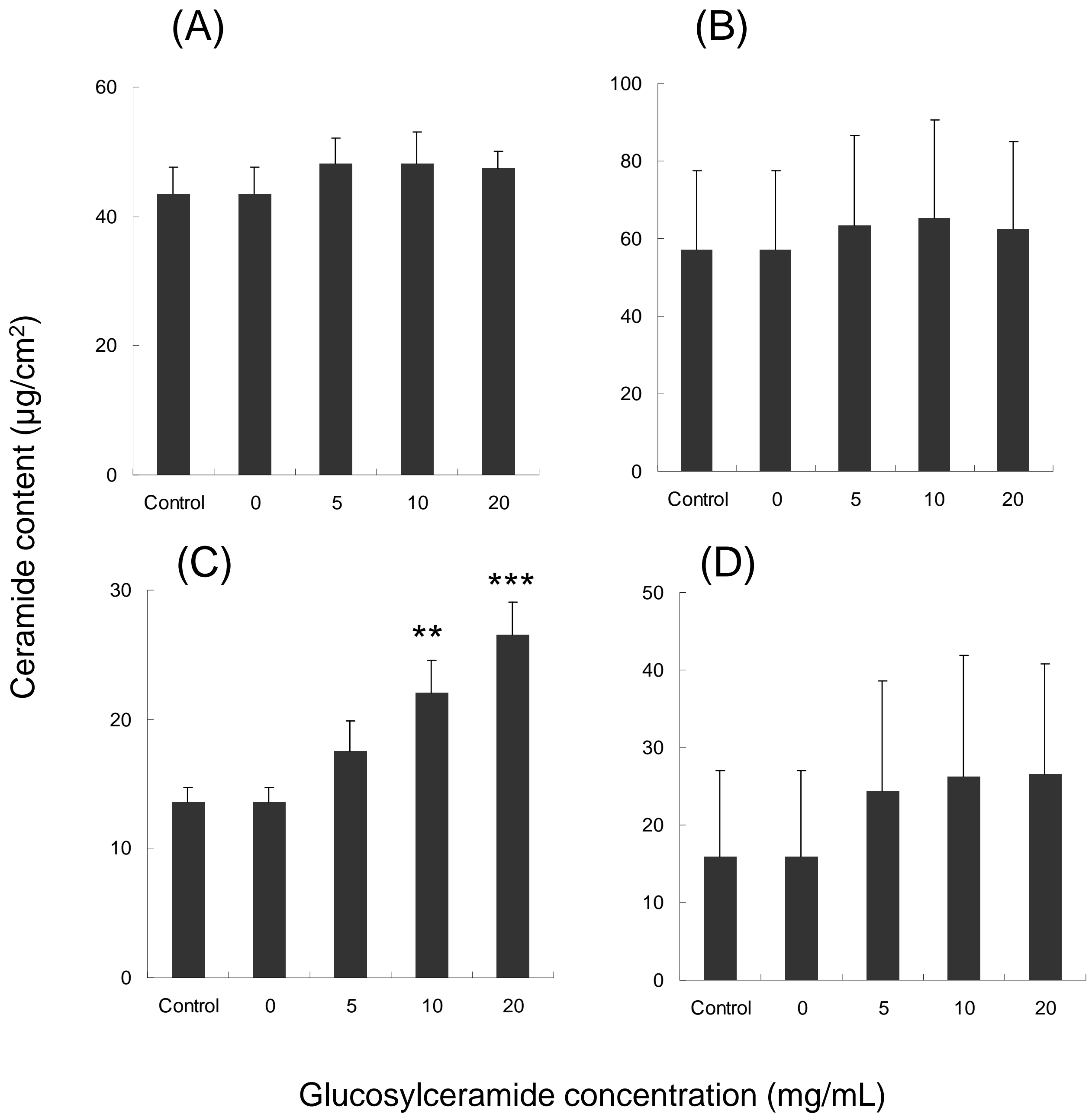

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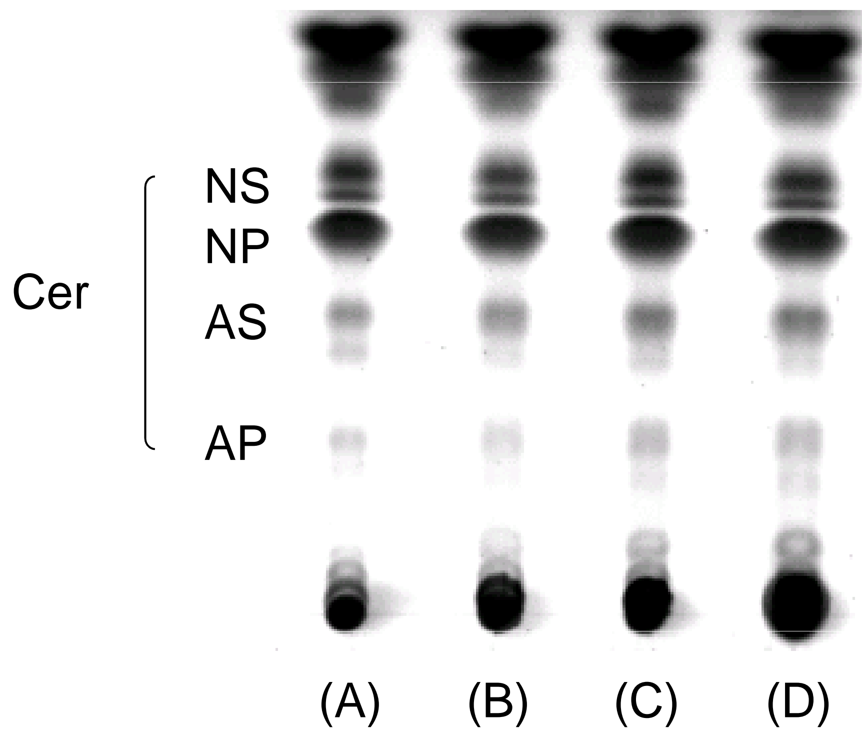

Figure 5

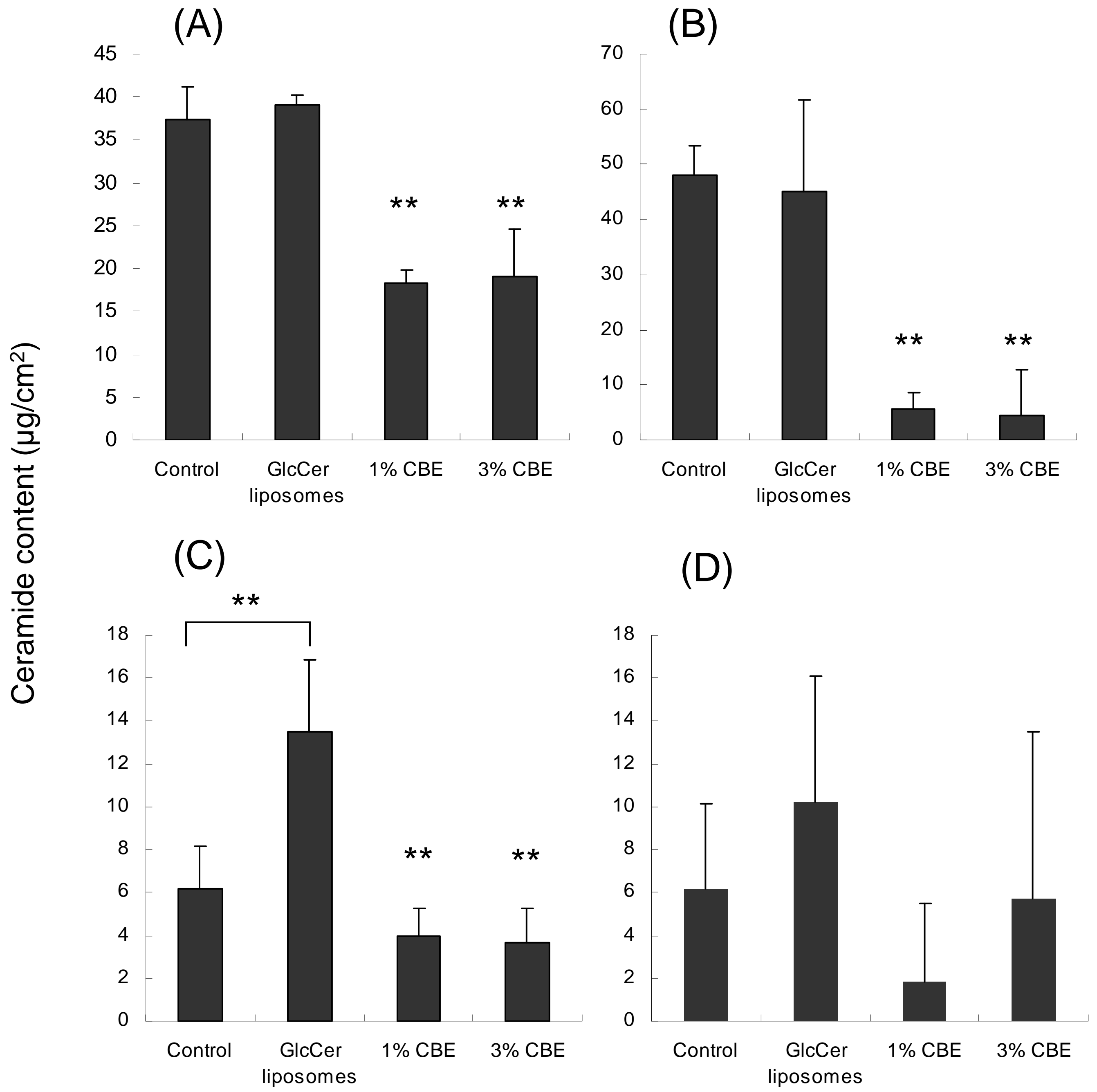

Figure 6