African Journal of Biotechnology Vol. 9(6), pp. 920-926, 8 February, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

The effect of phytosphingosine isolated from *Asterina pectinifera* on cell damage induced by mite antigen in HaCaT cell and antibacterial activity against *Staphylococcus aureus*

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Accepted 15 December, 2009

The current study was to investigate the positive protective effects of phytosphingosine (PS) against mite antigen and *Staphylococcus aureus*, etiological causes of an atopic dermatitis. To achieve this aim, PS was isolated from starfish, *Asterina pectinifera,* using high-performance liquid chromatography and was elucidated with nuclear magnetic resonance spectrometry. In the present experiment, PS, which ranged from 1 to 5 μM could protect the HaCaT cell against injuries caused by stimulation to 10 μg/ml mite antigen for 1 h, followed by incubation with serum-free medium for 24 h, which resembled the excitotoxin *in vivo* system. Furthermore, PS which was isolated from starfish could significantly inhibit the growth of *S. aureus*. In conclusion, this study demonstrated the protective effect of PS on excitotoxic damage against mite antigen and *S. aureus* through suppressing the excessive disruption of differentiation and exhibiting antibacterial capacity. This result implicated that the application of PS isolated from starfish might be a promising therapeutic option of atopic dermatitis.

Key words: *Asterina pectinifera*, atopic dermatitis, mite antigen, *Staphylococcus aureus*, antibacterial activity.

INTRODUCTION

Atopic dermatitis (AD) is known as an eczematous skin lesion which is caused by a complex interaction between environmental, genetic, immunological and biochemical factors with skin. At present, it is not possible to confirm which one is the primary initiator of AD, but most researchers have consistently noted the existence of a defective epidermal barrier (Ogawa and Yoshiike, 1993) and *Staphylococcus aureus* (*S. aureus*) infection in AD (Akiyama et al., 1996).

Phytosphingosine (PS) covalently binds to the corneocyte envelope and plays a crucial role in permeability barrier function. In addition, PS has gained attention as a major contributor to cutaneous antibacterial defence against bacterial toxins and infection both in its free form and as a part of the major fraction of ceramides. Nevertheless, little was known about the protective effects of PS against mite antigen-induced AD. Mite antigens have been reported to play an important role in the onset of AD (Furukawa et al., 2004), although little is known about the mechanism by which mite antigens trigger AD. Thus, the mite antigen was used as a causative agent of AD in HaCaT cells.

The HaCaT cell line is a useful candidate cell line for the *in vitro* mimicry system of AD (Lee et al., 2007). When grown in a serum-containing medium, HaCaT cells resemble precursors of human keratinocytes. Upon addition of the mite antigen which was extracted from *Dermatophagoides farinae* in house dust, cells gradually attained the phynotypic properties of AD (Pivarcsi et al., 2004).

S. aureus infection is another major contributor to the exacerbations of AD and shows resistance to existing therapy. Therefore, antibiotic therapy against *S. aureus* is an important component of treatment for AD, because it improves both the secondary infection and severity of AD

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(Jennifer et al., 2009). However, suppression of *S. aureus* has been poorly studied and is difficult to achieve. In contrast, although there is no direct evidence, the constitutive barrier disruption seen in the stratum corneum of patients with AD has been suggested to be associated with their predisposition to *S. aureus* colonization. This notion may lead to another important association between sphingolipid metabolites and the defense mechanism against *S. aureus* colonization because the barrier disruption has been ascribed to decreased levels of ceramides (Imokawa et al., 1991) in the stratum corneum, which appears to reflect possible altered sphingolipid metabolisms in the AD skin.

In spite of being unknown purified agent, there is very little or no data reported for the protective effects of PS isolated from starfish (starfish PS) against mite antigen-induced AD. In the present study, the starfish PS was investigated for its activity on HaCaT cell mite antigen-induced cell death and differentiation disruption. It was observed that starfish PS strongly promoted cell viability and highly induced cell differentiation in mite antigen-treated HaCaT cells. Furthermore, PS derived from starfish was also screened for its antibacterial activity against *S. aureus.* It was found that PS enhanced the protective effects and exhibited a remarkable activity against *S. aureus*.

MATERIALS AND METHODS

Chemicals

Immortalized human keratinocytes, HaCaT cells were purchased from American Type Culture Collection (Rockville, USA). Cell culture medium, or Dulbecco's Modified Eagle Medium (DMEM) and supplements were purchased from Invitrogen Corporation (New York, USA). Mite antigen was obtained from Yeonsei Medical School, Institute of Allergy (Seoul, Korea). Standard PS (95% purity) was obtained from Doosan Biotech (Kyunggi, Korea). Dimethylsulfoxide (DMSO) was purchased from Amresco (Ohio, USA) and Whatman filter papers were purchased from Whatman International Ltd (Maidstone, UK). All the other chemicals, unless otherwise indicated, were purchased from Sigma Chemical (St. Louis, USA). All the solvents in this experiment were of analytical grade.

Preparation of extract

Starfishes were collected from the seaside of the East Sea (Kyungpook, Korea). Collected starfishes were lyophilized and grinded into powders. The lyophilized starfish powder (50 g) was macerated and extracted with distilled water (1 l) at 4℃ for 6 h with stirring, then cleared by centrifugation (10,000 × g at 4℃ for 15 min). After repeating this procedure twice, the precipitation was soaked in distilled water (1 L) at 4℃ overnight with stirring. Following centrifugation (10,000 × g at 4℃ for 15 min), the crude water extract was further extracted by being soaked in an acetone solvent (1 l) at 4℃ for 24 h with stirring. The crude acetone extract was cleared by centrifugation (10,000 × g at 4℃ for 15 min). The supernatant was filtered through Whatman filter paper (5 μm) and the filtrate was concentrated using a vacuum rotary evaporator (Laborota 4000, Heidolph, Japan). The concentrate was lyophilized in a freeze-dryer (Neocool, Yamato, Japan) and stored at - 70℃ for further use.

Isolation of PS

The crude acetone solvent extract was separated using open column chromatography over silica gel (0.063 - 0.2 mm; Merck 7734, column size 5.0 × 20 cm), which was eluted with chloroform that contained 1% methanol, isocratically. The crude acetone solvent extract and standard PS were confirmed by silica gel (silica gel $60F_{254}$; Merck) thin layer chromatography using chloroform/methanol/ammonia in the ratio of 70:20:4 (v/v/v) as the solvent system. After air-drying at room temperature, the plates were examined under ultraviolet light. The spots of the unknown compounds were detected on the developed plate. Subsequently, retardation factor (RF) values of 0.3, 0.4 and 0.6 were evaluated. The selected spot of RF value (0.4) was approximately equal to the RF value of standard PS. For high-performance liquid chromatography (HPLC) analysis, the acetone solvent extract was dissolved in methanol as it was insoluble in ethyl acetate. Subsequently, the qualitative identification of standard PS and PS from starfish extract was performed through HPLC analysis. A mobile phase of acetonitrile and methanol was prepared in the ratio of 3:7 (v/v) and used as the eluent, isocratically. A C-18 reversed-phase column (particle size 4 μm; column size 3.9 × 300 mm) was used for 30 min at a flow rate of 1 ml/min. The column eluent was monitored by a UV detector at 254 nm. PS present in the extracts was identified by comparing its retention time with that of the standard PS sample. The relative concentrations of PS were calculated by HPLC using a standard calibration method. Using this method, all of the process-related substances were detected with good resolution.

Spectral analysis

The structure of the compound was identified according to spectroscopic analysis. ^{1}H-NMR data were acquired using an NMR spectrometer (Bruker, Avance 500 MHz) at the Korea Basic Science Institute. CD_3OD was used as a solvent. Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane, which was used as an internal reference.

Cell culture and treatment

Monolayers of HaCaT cell were grown in 100 mm culture dishes in DMEM medium-supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were main-tained in a humidified incubator equilibrated with 5% CO_2 at 37℃. When cells were grown to 70 - 80% confluence (day 1), some cells were seeded in 30 mm culture dishes at a density of 3.0×10^5 cells/dish in the serum-free medium and were pre-incubated for 24 h. Subsequently, the old medium was removed; cells were treated with a mite antigen in fresh serum-free medium under the appropriate conditions for 1 h. At the end of the incubation, the cells were transferred to another fresh serum-free DMEM medium with different concentration of standard PS, starfish PS and dexamethasone (Dexa.), then differentiation was induced for an additional 24 h.

For cell experiments, test compounds such as standard PS and starfish PS were dissolved in DMSO on the day of the experiment and then diluted with serum-free medium to obtain the appropriate concentration. Dexa. was dissolved in distilled water. Crude extract was filtered with a 0.22 μm syringe filter after it was dissolved in distilled water on the day of the experiment. Control groups were also performed in the absence of test compounds for the same incubation periods.

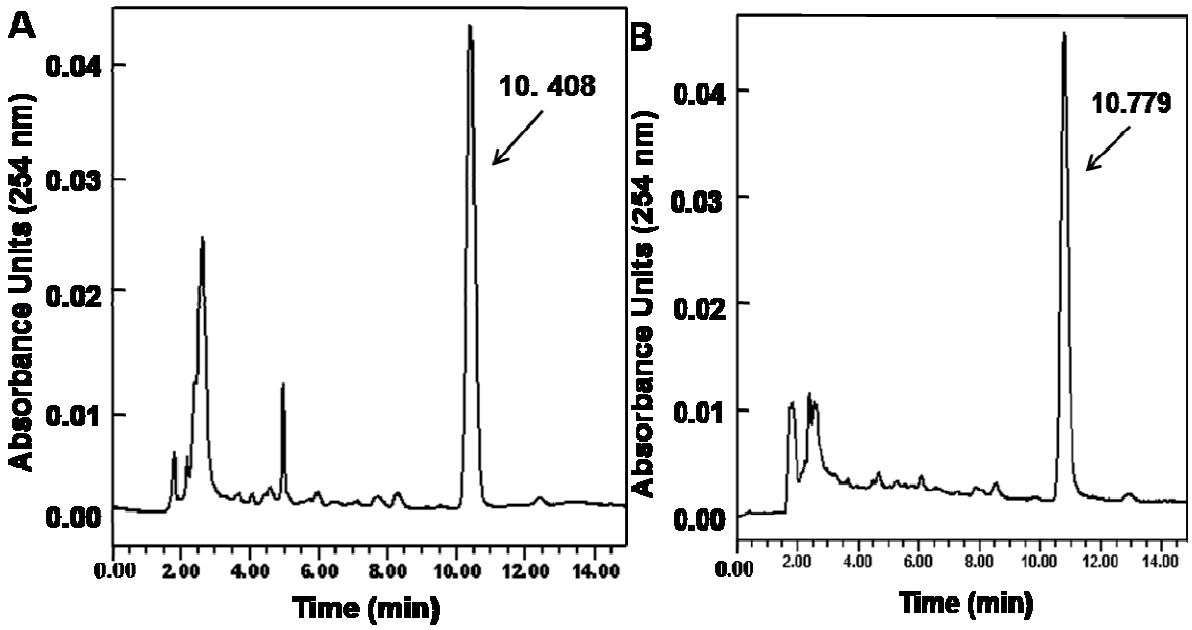

Figure 1. HPLC chromatograms of PS. A = Standard PS; B = PS isolated from *Asterina pectinifera*; HPLC column = C-18 (3.9 × 300 mm, 4 µm); Buffers = 3:7 (v/v) of acetonitrile and methanol; Detection = 254 nm; Flow rate = 1 ml/min; Column temperature = 25°C.

Cell viability determination

To determine cell viability, trypan blue dye exclusion and 3 -[4,5-dimethylthiazole-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assays were performed. HaCaT cells were grown in DMEM supplemented with 10% FBS, 100 unit/ml and 100 µg/ml streptomycin in a humidified incubator equilibrated with 5% CO_2 at 37°C. After 70 - 80% confluence (day 1), differentiation was induced and maintained onto a 96-well plate (5×10^4 cells/well) in serum-free DMEM medium before treatment with mite antigen. The cells were further stimulated in fresh serum-free DMEM medium for 24 h and subsequently treated with different concentration of mite antigen (2.5, 5, 10, 20 and 40 µg/ml) for 15, 30 and 60 min. Control cells were also grown in the absence of test sample for the same incubation periods. The cells were transferred to fresh serum-free DMEM medium with MTT solution. After incubation for 4 h at 37°C, the resulting supernatant was discarded and 150 µl solution of the same ratio of DMSO and EtOH was added. The optical density (OD) was immediately measured at 540 nm after the plates were shaken for 20 min. The ratios of viable cells were expressed in the form of a percentage of control OD as the mean ± standard deviation (SD) from n independent experiments, each assayed in triplicate. The combined data were compared using a one-way analysis of variance and the significance of the difference between the means. The combined data were compared using a one-way analysis of variance.

Antibacterial testing

S. aureus was maintained on nutrient agar and recovered by culturing in nutrient broth for 24 h. Bacterial culture was diluted 1: 100 with fresh sterile nutrient broth. Subsequently, the bacteria were streaked in radial patterns on the agar plates and were pre-incubated for 12, 18 and 24 h at 37°C with the purpose of examination for suppressive capacity of growth against *S. aureus*. The standard PS and starfish PS were tested at 0.0016, 0.016, 0.024 and 0.032 g/ml. Blank plates containing only nutrient agar and another set containing nutrient, dH_2O and DMSO served as controls.

Statistical analysis

Where applicable, the data were analyzed using a one-way analysis of variance and determined by Tukey's multiple range test ($p < 0.05$).

RESULTS AND DISCUSSION

Isolation of PS from starfish

HPLC chromatograms of both a standard solution containing PS and the starfish extract are shown in Figure 1. The retention time (10.779 min) of the largest peak in the starfish extract in Figure 1B was similar to that (10.408 min) of major peak in the standard as shown in Figure 1A. This was an indication that the compound extracted by the acetone solvent could be the same compound as the standard PS. The thicker the solution, the more stable the baseline after the major peak in the standard solution was shown (Figure 1A).

The structure elucidation through NMR

A number of methylene, methyl groups, hydroxyl groups and amino group of starfish and standard PS were detected

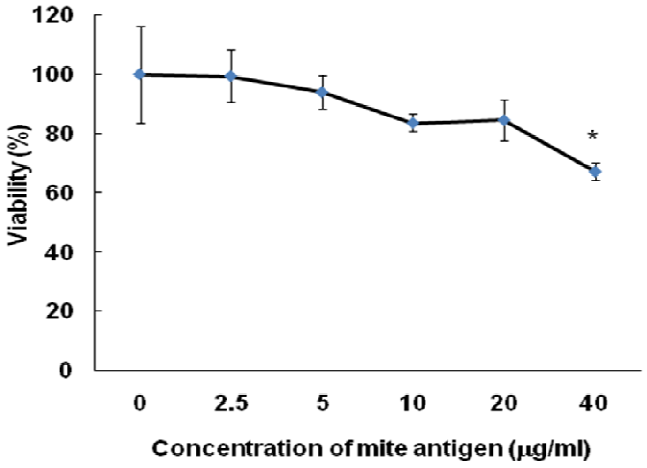

Figure 2. Cytotoxic effect of mite antigen in HaCaT cells. After HaCaT cells were exposed to increasing mite antigen concentrations (2.5 - 40 μg/ml) for 1 h, the cells were transferred to fresh medium without FBS. Then, cell viability was measured using MTT. Data shown were expressed as the mean ± SD, n = 3, *P < 0.05 analyzed by a one-way analysis of variance.

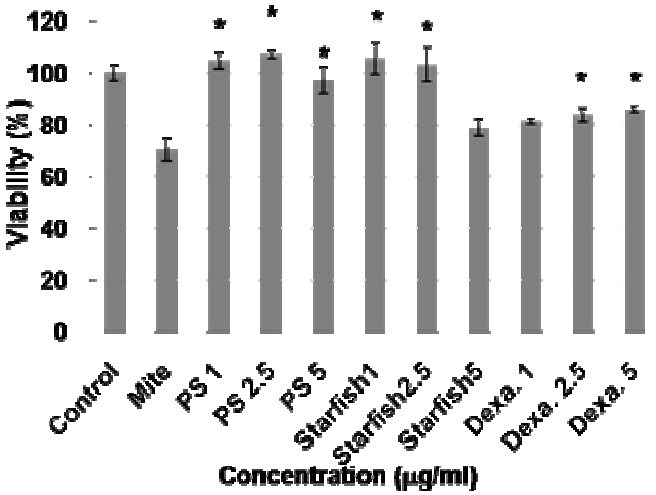

Figure 3. Cell viability effect of PS, starfish PS and Dexa. against mite antigen toxicity. HaCaT cells were treated with mite antigen 10 μM for 1 h, then cells were analyzed for cell viability using MTT after another incubation time of 24 h with different concentration of standard PS (PS), starfish PS and Dexa. (1 - 5 μM) in the fresh medium without FBS. Data shown were expressed as the mean ± SD, n = 3, *P < 0.05 analyzed by a one-way analysis of variance.

and assigned in the ^{1}H-NMR spectra (Spectra not shown). It was assumed that there were no significant or specific salvation shifts. The ^{1}H-NMR spectra of PS showed the methyl group of standard PS at δ 0.90 (3H, *t, J* = 7.0 Hz) with the relative number of protons of 9% and that of starfish isolate exhibited signals at δ 0.80 - 1.00 (13%). In methylene region, ^{1}H-NMR spectrum of stan-dard PS gave peaks (~ 74%) and that of starfish isolate gave less number of proton peaks (~ 40%) at δ 1.20 - 1.44). The difference between standard PS and starfish isolate in amino group was monitored. In comparison to standard PS, starfish isolate showed specific signals at δ 2.00 - 2.40 (14%) even though both of them showed the presence of amino group with almost same amount (~ 7%) at δ 1.50 - 1.80. The ^{1}H-NMR spectra of standard PS and starfish isolate in methanol gave multiplet spread over δ 3.2 - 3.8 (~ 3%). Although the result obtained indicated that the extract isolated from starfish was phytosphingosine. Further study will be needed in this regard to elucidate its accurate structure based on spectroscopic analysis.

Toxicity of mite antigen in cultured HaCaT cell

By using an MTT assay test upon stimulation of HaCaT cells with the mite antigen in the range of 2.5 - 40 μg/ml, dose-dependent mite antigen toxicity was measured after 1 h. As shown in Figure 2, mite antigen induced a decrease in cell survival in a dose-dependent manner, exceptionally after exposure to 20 μg/ml mite antigen. The result of 40 μg/ml mite antigen stimulation showed a significant ($P < 0.05$) decrease by 67.3%, whereas 10 and 20 μg/ml mite antigen treatments resulted in a reduction of approximately 18 and 15%, respectively. Although there was no significant injury in HaCaT cells after exposure to 10 μg/ml mite antigen, it was suggested that an 18% reduction could lead to the change in cell morphology and differentiation (Lee et al., 2007). Therefore, 10 μg/ml mite antigen was chosen as the test stimulant for its toxic effect.

Effect of PS on cell viability

The exposure of HaCaT cells to 10 μg/ml mite antigen for 1 h followed by incubation with serum-free medium for 24 h produced an obvious decrease in cell viability measured by MTT. Treated with different concentration (1 - 5 μM) susceptible to drugs such as Dexa., standard PS and starfish PS, the cell damage was investigated to show if the above agents had potential against the mite antigen or not. As shown in Figure 3, the mite antigen decreased the rate of cell viability by an approximate of 30%. After incubation in 1, 2.5 and 5 μM PS, PS exhibited a significant ($P < 0.05$) increment of cell viability by 104.6, 107.1 and 97.0%, respectively. This indicated that after exposure to the mite antigen, standard PS significantly increased the cell viability. In comparison to standard PS, starfish PS significantly increased the rate of cell viability by 105.3 and 103.0% at 1 and 2.5 μM, respectively. However, a 5 μM starfish PS resulted in only 9% viability increase after mite antigen-induced cell damage. It was

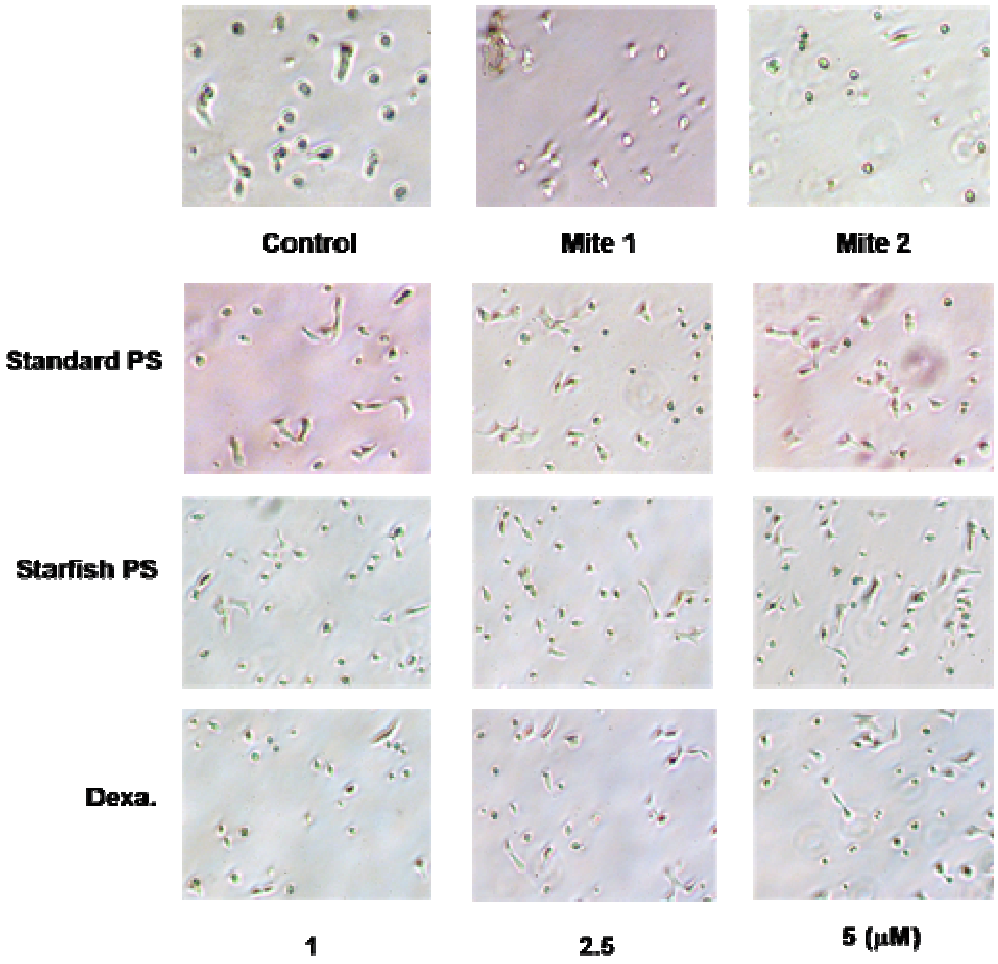

Figure 4. Effect of standard PS, starfish PS and Dexa. on mite antigen-induced change of cell morphology (× 250). HaCaT cells were pretreated with 10 μg/ml mite antigen for 1 h in serum-free medium. Subsequently, cells were treated with different concentrations of standard PS, starfish PS and Dexa. (1 - 5 μM) for 24 h. Mite 1 = after exposure time of 1 h to 10 μg/ml mite antigen; Mite 2 = after exposure time of 24 h to 10 μg/ml mite antigen.

suggested that the optimal concentration, or the balance between sphingoid bases (DHS/PHS/Sphingosine), their phosphorylated counterparts and ceramides could control the fate of the cells (Gailit and Clark, 1994).

Although, dexamethason is commercially used as a drug against AD (Furukawa et al., 2004; Kakinuma et al., 2001), still it showed little but significant increase in the effect on cell viability by 81.3, 83.9 and 85.5% in a dose-dependent manner. However, PS and starfish PS exhibited more remarkable increment of cell viability than that of Dexa.

Indeed, several constituents of house dust mites can cause a non-immune specific inflammatory reaction via their serine protease activity in conjunction with the elicitation of an allergen-specific response. Tryptic enzymes released by mite antigen can be exogenous activators which can also be importantly involved in the development of pruritus (Steinhoff et al., 2003). Furthermore, both enhanced serine protease activity as well as reduced ceramide expression in AD can reasonably provide a direct link through enhancement of several enzyme function (Hachem et al., 2005). These results suggested that starfish PS played a crucial role for cell viability by regulating various enzymes against mite antigen-induced dysfunction.

Suppressive effect on mite antigen-induced differentiation disruption

The cell morphology of HaCaT cells was observed by microscope after treatment with mite antigen and other agents for protection against mite antigen such as Dexa., standard PS and starfish PS. As shown in Figure 4, a control group revealed the disappearance of cellular processes and remarkable decrease of the differentiation

Table 1. Inhibitory activity of standard PS, starfish PS and Dexa. tested on bacteria by disk diffusion assay.

Sample	*Staphylococcus aureus*		
	12 h	18 h	24 h
dH2O	0[a]	0	0
DMSO	0	0	0
Standard PS	0	0	0
Starfish PS	14.5 ± 0.50[b]	12.5 ± 0.87	11.9 ± 0.69
Dexa.	0	0	0

1×10^7 cfu/dish of bacteria was spread on TSA; 50 µl of starfish PS/disk was investigated. Plates were incubated for 24 h at 37°C.
[a]No clear zone produced;
[b]Mean ± SD (mm) of diameter of clear zone including diameter of disk (8 mm) from three experiments.
Significantly different from starfish PS and the others at $P < 0.05$.

although it contained a high percentage of cell viability. There was an injury in HaCaT cells after treatment with 10µg/ml mite antigen for 1 h exhibiting the disappearance of cellular processes, decrease of the differentiation and increase of the disintegration. In addition, the mite antigen strongly increased cell death after 1 day. However, the damage in groups of standard PS and starfish PS-treated cells was markedly decreased. Furthermore, the cells in those groups activated obvious differentiation against mite antigen-induced injury. But when the cells were exposed to higher than the optimum concentrations of starfish PS or the cells were in prolonged incubation, the viable rate was decreased due to cytotoxic effect. In comparison with results of standard PS and starfish PS, the change in cell morphology in Dexa. showed evident differentiation activity at 1-5 µM even though cell viability was expressed in the form of a slight percentage, yet significantly.

The existence of a defective permeability barrier function in the skin of AD patients is well accepted and the epidermal abnormality, generally viewed in the past as a consequence of the inflammatory phenotype, is considered the outcome of a pre-existing defect of epidermal differentiation (Elias, 2005). Epidermal differentiation leads to the formation of the stratum corneum (SC) (Feingold et al., 1991). Much of permeability barrier function is provided by the CE (Elias, 2005) resided on the exterior of the dead cornified cells, SC and the disturbance of a skin barrier may result in AD. Among the backbones of ceramides (Hannun, 1996; Kim et al., 2006), the physiological functions of PS in the epidermis are still poorly known. However, it has been shown that the amount of newly synthesized protein-bound lipids in the CE was significantly reduced in atopic affected regions (Clark, 1993). Based on this study, it is plausible that standard PS and starfish PS maintain the amount of newly synthesized protein-bound lipids in mite antigen-induced disruption via some enzyme activities, then facilitate the epidermal differentiation.

Antimicrobial activity of PS isolated from starfish

The antimicrobial activity of standard PS, starfish PS and Dexa. against *S. aureus* is shown in Table 1. In comparison with the effect on HaCaT cell, a body of evidence was not reported that standard PS could diminish the growth of *S. aureus* under the test conditions employed. Although, Dexa. was also subjected to the antimicrobial activity assay, still there was no protective effect up to the maximum soluble concentration 2.5 mM. However, only starfish PS showed a significant antibacterial activity after the performed periods of 12 to 24 h under the test conditions. Starfish PS (0.032 g/ml) markedly inhibited the growth of *S. aureus* from 11.9 ± 0.69 to 14.5 ± 0.50 mm (increased by 145%). According to a previous study (Junko et al., 2002), the downregulated production of sphingosine and its relevance for increased colonization of AD skin through skin surface bacteria including *S. aureus* suggested that sphingosine participates in the innate defense mechanism against the proliferation of *S. aureus* in the SC of healthy control skin. Thus, the deficiency of sphingosine may be involved in the high incidence of *S. aureus* colonization seen on the AD skin. Indeed, one sphingolipid metabolite, sphingosine, but not ceramide, is well known to have potent antimicrobial effects on *S. aureus* at physiological levels (Bibel et al., 1992b, 1993).

Various arguments (Gandhi and Cherian, 2000; Takechi et al., 2003) lead to the hypothesis that the positively charged sphingosine is attracted to the negatively charged bacterial cell at physiological pH, by electrostatic attraction and hydrophobic interactions and after reaching the cytoplasmic membrane, it forms channels as the long hydrophobic chain penetrates into the lipid bilayer. Being positively charged, sphingosine shows high structural similarity with quaternary ammonium compounds. The mode of action of the latter is based on adsorption to the cell surface, diffusion through the cell wall and disruption of the cytoplasmic membrane. This probably leads to

secondary leakage of metabolites and enzymes, killing the bacteria (Merianos, 2001; Paulus, 1993). The current result suggested that starfish PS had an evident effect on penetration through bacterial cell body and protected cell growth via various pathways. However, further assays for antimicrobial susceptibility test and the multiple regulatory roles of starfish PS against *S. aureus* will be needed.

Conclusion

The results in this study have elucidated that standard PS and starfish PS could increase significant cell viability and elicit remarkable differentiation activity on mite antigen-induced disruption in HaCaT cells. In comparison study, starfish PS markedly and significantly prevented the growth of *S. aureus*, whereas the inhibitory effect of PS towards the *S. aureus* activity was rarely under the test conditions. In conclusion, these results provided an insight into starfish PS-mediated multiple signals in epidermis homeostasis and moreover pointed to the promising use of starfish PS as a therapeutic agent in the treatment of AD.

ACKNOWLEDGEMENTS

This research work has been performed under the BK21 project, Korea. The author thanks Doosan Corporation Biotech Bu for providing PS.

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