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Caspase-14 Activation by *Chaenomeles sinensis* May Enhance Skin Barrier Function

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Abstract

Atopic dermatitis (AD) is characterized by chronic itching and significant loss of skin barrier function. Decreased filaggrin levels have been observed in several patients with AD and are thought to be responsible for impaired barrier function. Caspase-14 is an important factor in maintaining skin barrier function and is involved in the production of natural moisturizing factors by degrading filaggrin. We have been conducting research on *Chaenomeles sinensis* extract (CSE) and confirmed its anti-aging effects. In this study, we investigated the effects of CSE on caspase-14 expression. CSE-induced changes in gene expression were confirmed in human keratinocytes using DNA microarray and quantitative polymerase chain reaction (qPCR), and the expression of caspase-14 increased. This was confirmed at the protein level using western blotting. Furthermore, CSE increased the expression of kallikrein (KLK) 5 and KLK7, which are involved in skin barrier function, as well as caspase-14. In the investigation of the activation of these factors, CSE promoted activation of caspase-14, KLK5, and KLK7. Moreover, procyanidin B2, gallocatechin gallate, and quercetin were identified using HPLC as CSE components, confirming the strong promotion of caspase-14 activity by quercetin. These results suggest that CSE effectively improves skin barrier function by promoting the expression and activation of caspase-14.

Keywords

Skin Barrier Function, Caspase-14, KLK5, KLK7, *Chaenomeles sinensis*

1. Introduction

Atopic dermatitis (AD) is a chronic skin disease characterized by itchy eczema and significant loss of skin barrier function. Approximately 30% of patients with AD have filaggrin gene abnormalities; however, there may be other causes of skin

barrier dysfunction [1]-[4]. The production of natural moisturizing factor (NMF) production involves the degradation of profilaggrin by kallikrein (KLK) 5 and skin aspartic protease (SASPase) to produce filaggrin, which is fragmented by caspase-14 and degraded to the amino acid level by calpain-1 and bleomycin hydrolase to produce NMF [5]-[8]. Caspase-14 deficient mice have been reported to accumulate filaggrin and exhibit increased transepidermal water loss (TEWL), suggesting that caspase-14 is an important factor in maintaining skin barrier function [9].

We previously studied *Chaenomeles sinensis* extract (CSE) and confirmed that it has superoxide dismutase (SOD)-like activity, inhibits collagenase activity, and inhibits elastase activity [10]. *C. sinensis* is a species native to China. CSE is listed in the Japanese standards for quasi-drug ingredients, and the plant is a popular raw material for cosmetics and quasi-drugs. In addition to its anti-aging effects, CSE has been shown to have anti-tumor, anti-hepatitis, anti-bacterial, immunomodulatory, and anti-influenza varus effects [11]-[13]. Furthermore, significant inhibition of scratching behavior by the pruritogenic compound 48/80 has been reported and is expected to be effective in the treatment of AD [14]. The active constituents of *C. sinensis* have been reported to be phenols (vanillic acid, gallic acid, chlorogenic acid, ferulic acid, p-coumaric acid), triterpenes (oleanolic acid, ursolic acid) and flavonoids (rutin, catechin, epicatechin, quercetin, procyanidin) [15]-[17]. Epigallocatechin gallate, a type of catechin, has been reported to promote the production of filaggrin and caspase-14, and CSE is expected to improve skin barrier function [18]. Therefore, we investigated the effects of CSE on caspase-14 production and activation.

2. Materials and Methods

2.1. Reagents

Ascorbic acid and quercetin were obtained from FUJIFILM Wako Pure Chemicals (Osaka, Japan). Gallic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Gallocatechin, catechin, epigallocatechin gallate, gallocatechin gallate, epicatechin gallate, and catechin gallate were purchased from Nagara Science (Gifu, Japan). Procyanidin B2 was purchased from Extrasynthese (Lyon, France). All the other chemicals were purchased from FUJIFILM Wako Pure Chemicals.

2.2. Plant Material and Extraction

In the present study, we used *Chaenomeles sinensis* Koehne (*Rosaceae*) obtained from Nara, Japan. The whole fruit of *C. sinensis* was dried, powdered, and extracted with water, ethanol, and 1,3-butylene glycol (BG). CSE concentrations were calculated in terms of solid content concentration and used in the assay.

2.3. Cell Culture and Treatment

The human keratinocyte cell line HaCaT was obtained from AddexBio Technologies (San Diego, CA, USA) and cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 25 mM

glucose. Normal human keratinocytes (NHEK) were obtained from Kurabo (Osaka, Japan) and the cells were grown in keratinocyte growth medium (KGM) (Kurabo) at 37˚C under a humidified atmosphere containing 5% CO_2.

2.4. DNA Microarray Analysis

NHEK were seeded in 12-well plates at a density of 2.5×10^5 cells and cultured in KGM for 24 h, followed by treatment with keratinocyte basal medium (KBM) containing CSE at 50 μg/ml. After 24 h, the cells were lysed with the QIAzol Lysis Reagent. The miRNeasy Mini Kit (QIAGEN, Venlo, Netherlands) was used to purify the RNA, and DNA microarray analysis was performed using Clariom S (Thermo Fisher Scientific, Waltham, MA, USA). Data analysis was performed using Transcriptome Viewer (Kurabo). The expression level of each gene was expressed as a ratio of the expression level without CSE (control) to 1.

2.5. Reverse Transcription—Quantitative Polymerase Chain Reaction

NHEK were seeded in 48-well plates at a density of 5×10^4 cells and cultured in KGM for 24 h, followed by treatment with KBM containing CSE. The cells were harvested and total RNA was isolated using the CellAmp Direct RNA Prep Kit for Reverse transcription-quantitative polymerase Chain reaction (RT-qPCR) (TaKaRa Bio, Shiga, Japan) according to the manufacturer's instructions. Purification of the first-strand cDNA and RT-PCR were performed using One Step TB Green PrimeScript RT-PCR Kit II (TaKaRa Bio) to prepare the reaction mixture. The thermal cycle profile was as follows: reverse transcription phase for 5 min at 42˚C followed by the hot start phase at 95˚C for 10 s and 50 cycles of amplification at 95˚C for 5 s and 60˚C for 30 s. GAPDH was used as the internal control. All the primers were obtained from Sigma-Aldrich. The control group did not receive CSE treatment.

2.6. Western Blot Analysis

HaCaT cells were seeded in 24-well plates at a density of 8×10^4 cells and cultured in DMEM for 24 h, followed by treatment with the assay medium (DMEM supplemented with 0.25% FBS and 25 mM glucose) containing CSE. The cells were harvested and lysed in Triton-lysis buffer (PBS with 0.5% Triton-X, protease inhibitor cocktail (Abcam, Cambridge, UK)). After removal of insoluble cell debris, 20 μg of protein were electrophoresed through a 12% polyacrylamide gel and blotted onto a PVDF membrane (Amersham Pharmacia Biotech, Amersham, UK). Membranes were blocked for 1 h in blocking buffer (PBS with 3% skim milk, 0.1% Tween 20) and incubated with HRP conjugated caspase-14 specific antibody (Abcam) diluted 1:1000 in blocking buffer overnight at 4˚C. β-actin (Abcam) was used as a loading control.

2.7. Kallikrein Activity Assay

HaCaT cells were seeded in 24-well plates at a density of 8×10^4 cells and

cultured in DMEM for 24 h, followed by treatment with the assay medium containing CSE. KLK activity was measured using Boc-VPR-7-methoxycoumarin-4-acetic acid (MCA) (KLK5) (Peptide Institute, Osaka, Japan) and Suc-LLVY-MCA (KLK7) (Peptide Institute) as substrates. The assay buffer consisted of 25 mM Tris-HCl buffer, 5 mM ethylenediaminetetraacetic acid and 10 mM dithiothreitol (DTT). Cells were suspended in KLK assay buffer and lysed by three freeze-thaw cycles with liquid nitrogen. The lysate was centrifuged at 13,000 × *g* for 10 min, and the resulting supernatant was diluted 1/5 with the assay buffer. The diluted solution and substrate solution were mixed 1:1 and reacted at 37˚C for 1 h. The KLK activity was measured at excitation and emission wavelengths of 380 and 460 nm, respectively. The control group did not receive CSE treatment.

2.8. Caspase-14 Activity Assay

HaCaT cells were seeded in 24-well plates at a density of 8×10^4 cells and cultured in DMEM for 24 h, followed by treatment with the assay medium containing CSE or CSE components. Caspase-14 activity was measured using Ac-WEHD-MCA (Peptide Institute) as the substrate. The assay buffer consisted of 1.3 M sodium citrate buffer and 5 mM DTT. Cells were suspended in caspase assay buffer and lysed by three freeze-thaw cycles with liquid nitrogen. The lysate was centrifuged at 13,000 × *g* for 10 min, and the resulting supernatant and substrate solution were mixed 1:1 and allowed to react at 37˚C for 1 h. The activity of caspase-14 was measured at the excitation and emission wavelengths of 380 and 460 nm, respectively. The control group did not contain any samples.

2.9. Determination of components by HPLC

HPLC was performed using a HITACHI 5420 UV-VIS detector (Hitachi High-Tech, Tokyo, Japan). Separation was conducted using Luster C18 (5 μm, 4.6 × 150 mm, Dikma Technologies, Foothill Ranch, CA, USA). The solvents used for analysis were 0.1% formic acid (A) and acetonitrile (B). Two columns were used in series for quercetin detection, with a flow rate of 1 ml/min and a column temperature of 40˚C. The gradient program was 5 min from the start with 5% B, and then changed to 20% B at 45 min, 99% B at 48 min, and 5% B at 50 min. UV detection was performed at a wavelength of 370 nm. For components other than quercetin, the analyses were performed using a single column. The gradient program was for 1 min from start with 1% B and then changed to obtain 10% B at 6 min, 20% B at 30 min, 35% B at 45 min, 99% B at 48 min and 1% B at 50 min, with a flow rate of 1 ml/min and a column temperature of 40˚C. UV detection was performed at 280 nm.

2.10. Statistical Analysis

The experimental data were evaluated for statistical significance using Student's *t*-test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. CSE Enhances Caspase-14 mRNA Expression

To confirm the effect of CSE on skin barrier function, RNA was extracted from CSE-treated NHEK cells, and changes in mRNA expression were analyzed by DNA microarray. As shown in **Figure 1(a)**, the expression of proteases involved in stratum corneum differentiation, such as KLK, calpain, and caspase, was increased, whereas the expression of SPINK5, encoding LEKT1, a known inhibitor of KLKs, was suppressed. Notably, the expression of KLK5, KLK7, and caspase-14 increased by more than 2-fold.

Furthermore, RT-qPCR was used to analyze changes in the expression of caspase-14, an important factor in the enhancement of skin barrier function by CSE. mRNA expression levels were measured by adding CSE to NHEK at concentrations of 20, 50, and 100 μg/ml. As shown in **Figure 1(b)**, caspase-14 expression increased with increasing CSE concentration.

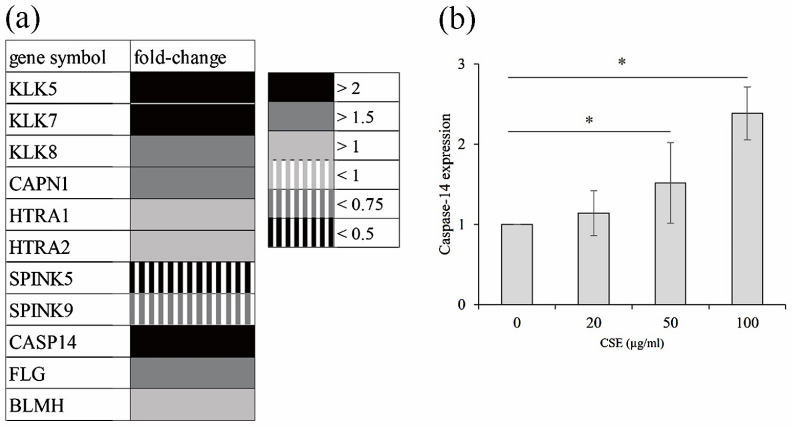

Figure 1. Effect of CSE on mRNA expression. (a) NHEKs were treated with 50 μg/ml of CSE and mRNA expression was comprehensively analyzed using DNA microarray analysis. (b) HaCaT cells were treated with 20, 50, and 100 μg/ml of CSE, and mRNA expression was analyzed comprehensively using RT-qPCR. All the data are expressed as the means ± SD of an experiment (n = 3 - 7). * $p < 0.05$.

3.2. CSE Enhances Caspase-14 Protein Expression

Using western blotting, we confirmed whether the increased expression of caspase-14 induced by CSE was also induced at the protein level. Protein levels were confirmed by adding 17 and 170 μg/ml of CSE to HaCaT cells, extracting the protein, and western blotting. Proteins were extracted after cells were grown until confluent and differentiated, because it has been reported that protein expression of caspase-14 is not induced in undifferentiated cells. The specificity of caspase-14 recognizing antibody was confirmed by the presence of a band at the same position as that of recombinant caspase-14.

As shown in **Figure 2**, HaCaT cells without the sample showed only a few bands, even after 72 h. Calcitriol, added as a positive control, showed the expression of

caspase-14 after 48 h of exposure or longer, with an increase in expression with longer exposure time. Exposure to CSE also resulted in the expression of caspase-14 after more than 48 h of exposure; caspase-14 expression was higher when exposed to 17 μg/ml than 170 μg/ml of CSE.

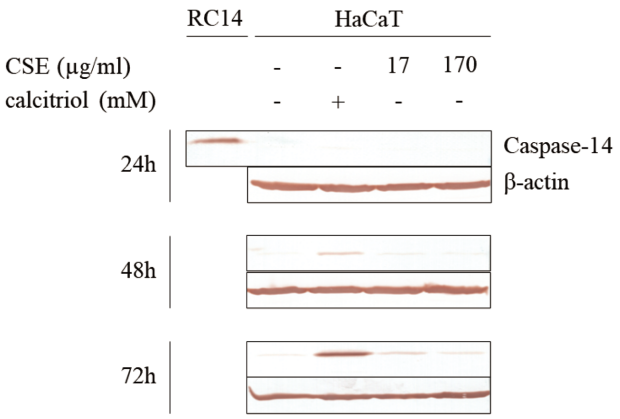

Figure 2. Effect of CSE on protein expression. HaCaT cells were incubated with calcitriol or CSE for 24, 48 and 72 h. Caspase-14 and β-actin expression levels were determined using western blotting. Recombinant caspase-14 (RC14) was used to confirm the band position.

3.3. CSE Enhances Caspase-14 and Kallikreins Activity

CSE enhances the expression of caspase-14. Therefore, we investigated the effects of CSE on caspase-14 activity. Proteins were extracted from HaCaT cells and incubated with the synthetic tetrapeptide substrate, Ac-WEHD-MCA. Treatment of HaCaT with 17 and 170 μg/ml of CSE tended to enhance the activity of caspase-14 (**Figure 3**). Notably, 17 μg/ml showed significant activity enhancement at 48 h of exposure.

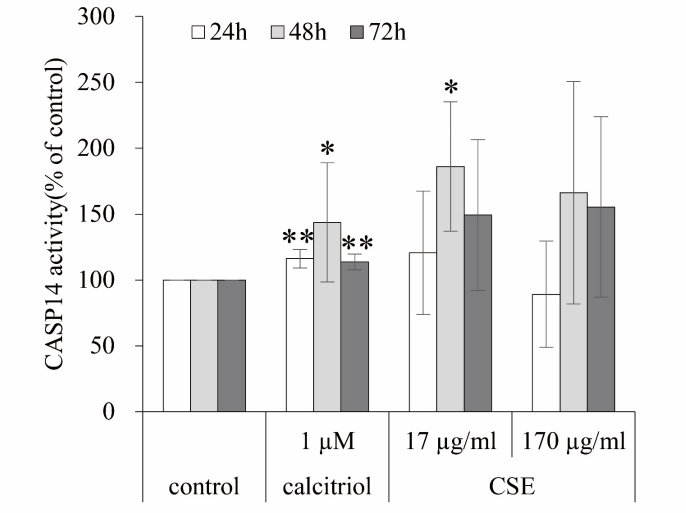

Figure 3. Effect of CSE on enzyme activation. HaCaT cells were incubated with calcitriol or CSE for 24, 48 and 72 h. Caspase-14 activity was measured using specific substrates. All the data are expressed as the means ± SD of an experiment (n = 4). * $p < 0.05$ and ** $p < 0.01$ compared with control at each time.

Chymotrypsin has been reported to be involved in the activation of caspase-14, with KLK7 being the most likely candidate. KLK5 and also a factor involved in the NMF production cascade along with caspase-14. Therefore, we evaluated KLK5 and KLK7 activation following CSE treatment. Proteins were extracted from HaCaT cells and incubated with the synthetic peptide substrates Boc-VPR-MCA or Suc-LLVY-MCA. KLK5 was accelerated by CSE at 17 and 170 μg/ml for all treatment times, while KLK7 was accelerated by CSE at 17 μg/ml for 24 and 48 h (**Figure 4(a)** and **Figure 4(b)**). KLK7, which is predicted to be involved in caspase-14 activation, was induced by CSE at an earlier stage than caspase-14.

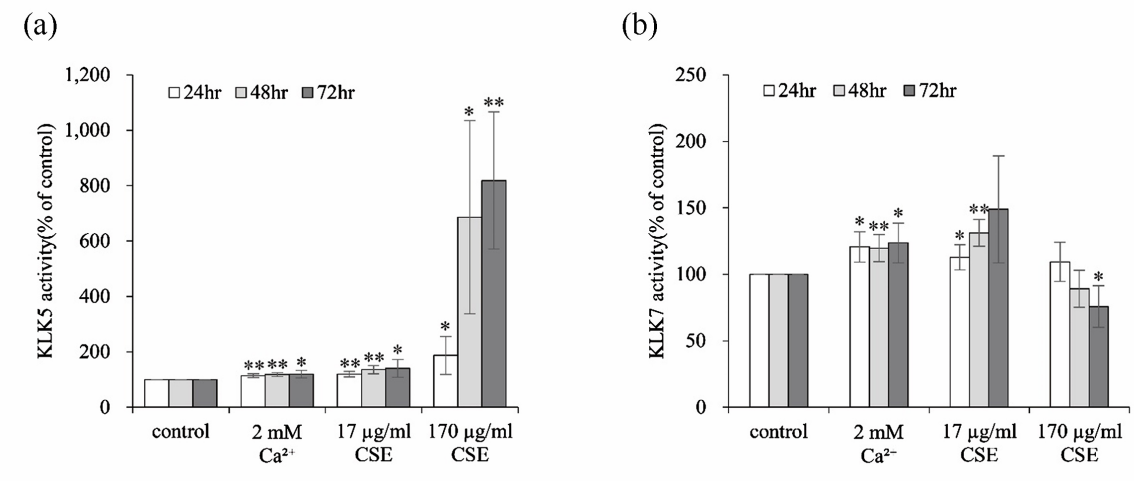

Figure 4. Effect of CSE on KLK activation. HaCaT cells were incubated with Ca^{2+} or CSE for 24, 48 and 72 h. (a) KLK5 and (b) KLK7 activities were measured using specific substrates. All the data are expressed as the means ± SD of an experiment (n = 4). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with control at each time.

3.4. Compositional Determination of CSE

CSE promotes the activation of caspase-14, KLK5, and KLK7. *C. sinensis* fruits are rich in triterpenes and phenolic compounds, including catechins and procyanidins, and we hypothesized that these components might be the active ingredients. Therefore, we determined the active components of CSE using HPLC to identify them (**Figure 5**). Peaks were characterized by comparing their retention times and UV spectra to those of standards. The results showed that 1 g of CSE contained 1275.1 μg of procyanidin B2, 383.9 μg of gallocatechin gallate, and 383.8 μg of quercetin.

3.5. Caspase-14 Was Activated by Procyanidin B2, Gallocatechin Gallate, and Quercetin

To identify the active ingredients in CSE, we investigated the effects of its components on caspase-14 activity. Proteins were extracted from HaCaT cells and incubated with the synthetic tetrapeptide substrate, Ac-WEHD-MCA. When HaCaT cells were treated with procyanidin B2, gallogatechin gallate, and quercetin, caspase-14 activity was significantly enhanced (**Figure 6**). In particular, quercetin showed enhanced activity at both 48 and 72 h, despite the lower treatment concentration.

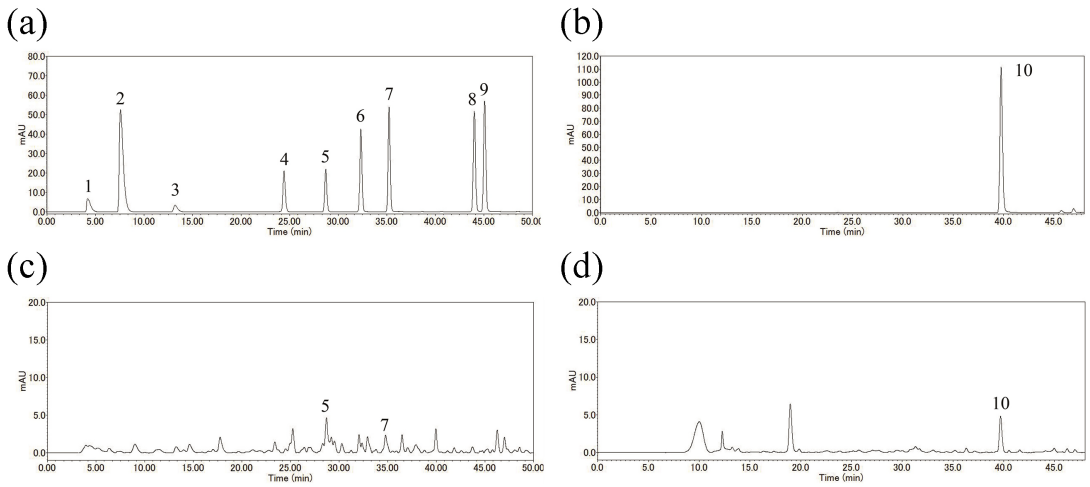

Figure 5. Compositional determination of CSE. Representative HPLC chromatograms of CSE. Chromatographs were taken at (a) (c) 280 nm and (b) (d) 370 nm are shown. (a) (b) standards, (c) CSE, (d) acid-treated CSE. 1: ascorbic acid (RT = 4.18; stabilizer), 2: gallic acid (RT = 7.57), 3: Gallocatechin (RT = 13.20), 4: catechin (RT = 24.44), 5: procyanidin B2 (RT = 28.75), 6: epigallocatechin gallate (RT = 32.35), 7: gallocatechin gallate (RT=35.27), 8: epicatechin gallate (RT = 44.05), 9: catechin gallate (RT = 45.12), 10: quercetin (RT = 39.57).

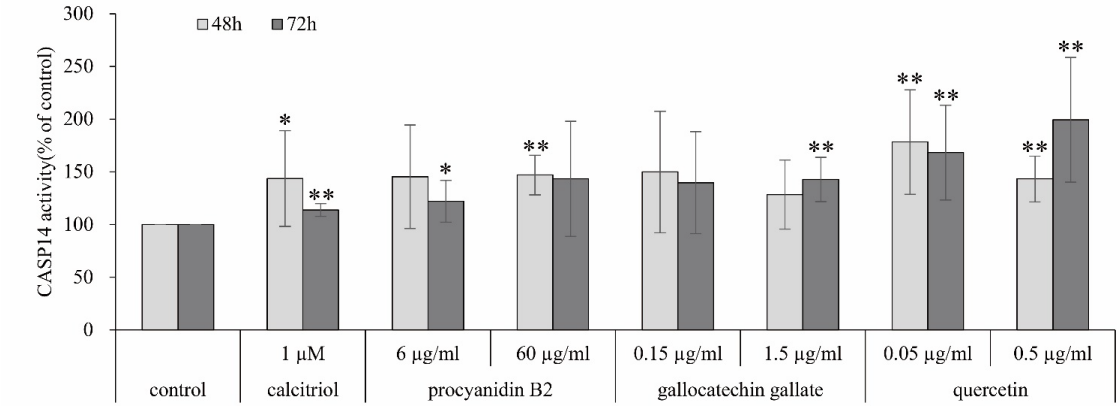

Figure 6. Effect of CSE on enzyme activation. HaCaT cells were incubated with calcitriol or CSE for 24, 48 and 72 h. Caspase-14 activity was measured using specific substrates. All the data are expressed as the means ± SD of an experiment (n = 4). *$p < 0.05$ and **$p < 0.01$ compared with control at each time.

4. Discussion

CSE has antioxidant, antibacterial, and anti-inflammatory properties and has been reported to effectively reduce allergic scratching [12] [14]. Epigallocatechin gallate, which is listed as a component of *C. sinensis*, has been reported to promote the production of filaggrin and caspase-14, and CSE is expected to improve skin barrier function [18].

Normalization of epidermal turnover and improvement of moisture retention are important for strengthening the skin barrier function. In keratinocytes, the expression of the cell-shedding enzymes KLK5 and KLK7 was increased by CSE, whereas the expression of SPINK5, which encodes LEKT1, an inhibitor of KLKs, was decreased. In terms of the factors involved in NMF production, there was no

change in the expression of filaggrin or bleomycin hydrolytic enzymes; however, there was an increase in the expression of calpain-1 and caspase-14. These findings suggest that CSE promotes desquamation and NMF production. Caspase-14 is known to be involved in NMF production by degrading filaggrin in the epidermis, and it has been reported that in caspase-14 deficient mice, filaggrin accumulates, NMF levels are reduced, and TEWL is increased [9]. caspase-14 is thought to be involved in keratinocyte denucleation [19]. Therefore, promoting the expression of caspase-14 may help maintain a healthy stratum corneum by enhancing the water-retention capacity of the skin and promoting normal epidermal differentiation. To investigate the expression of caspase-14 in more detail, we quantified its mRNA expression by RT-PCR. The results showed that caspase-14 expression increased in a CSE concentration-dependent manner. Furthermore, a time-dependent CSE-induced caspase-14 protein expression was observed. However, the protein expression was higher at lower concentrations, suggesting that protein expression may be post-transcriptionally regulated.

Although the caspase family is activated by the cleavage of procaspases by other caspases, caspase-14 is reported to undergo cleavage by chymotrypsin rather than by other caspase families, which is a critical step in its activation [20]. CSE promotes the activity of caspase-14, KLK5, and KLK7 in keratinocytes. KLK7 is the major chymotrypsin in the stratum corneum. Additionally, KLK7 is activated by the cleavage of KLK5 [21]. Thus, CSE may activate KLK7 by promoting KLK5 activity and caspase-14 by promoting the cleavage of procaspase-14 by KLK7.

The CSE used in this study contained procyanidin B2, gallocatechin gallate and quercetin. These components promoted caspase-14 activity in keratinocytes. Quercetin promoted caspase-14 activity at concentrations as low as the nanogram level. Based on these facts, we can say that gallocatechin gallate, quercetin, and procyanidins are good candidates for active ingredients in CSE. Epigallocatechin gallate, an epimer of gallocatechin gallate, promotes the production of filaggrin and caspase-14, which is consistent with our results [22]. Quercetin has also been reported to affect inflammation, oxidative stress, and damage healing in AD keratinocyte models [23]. Based on these results and the findings of the present study, quercetin is expected to be effective in the treatment of AD. CSE, it was a mixture, showed significant caspase-14 promoting activity only at 48 h exposure of 17 μg/mL, whereas quercetin showed significant promoting activity at all concentrations and exposure times. It is believed that CSE contains other compounds in addition to the three components identified in this study. It is assumed that extracts are more prone to variations in results from test to test than single components, because of the complex interplay of the effects of multiple components. Taken together, these results suggest that CSE is effective in enhancing turnover and NMF production, and strengthening skin barrier function by promoting the expression and activation of caspase-14, KLK5, and KLK7 in keratinocytes. Furthermore, it is suggested that the active components of caspase-14 activation in CSE are procyanidin B2, gallocatechin gallate, and quercetin.

Authors' Contributions

Yu Sumiya: Conceptualization, Methodology, Investigation, Data curation, Writing-original draft. Manami Yamaga: Methodology, Investigation. Keita Shigeyama: Supervision, Review. Ikuyo Sakaguchi: Supervision, Writing-review & editing.

Conflicts of Interest

Yu Sumiya, Manami Yamaga, Keita Shigeyama and Ikuyo Sakaguchi are employees of Club Cosmetics Co., Ltd.

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