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Eucalyptus Citriodora Extract Regulates Cutaneous Homeostasis Including Immune Dysregulation and Skin Barrier Dysfunction Via the Modulation of Peroxisome Proliferator-Activated Receptor-β/δ (PPAR-β/δ) Pathway

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ABSTRACT

Perturbation of cutaneous homeostasis including immune dysregulation and skin barrier dysfunction evokes skin disorders. In this study, we examined the effect of *Eucalyptus citriodora* (Euc-c) extract on cytokine production, cell proliferation and cell differentiation in HaCaT cells to elucidate its influence on cutaneous homeostasis. Euc-c suppressed significantly LPS-induced IL-6 and TNF-α-induced IL-8 production from HaCaT cells. Conversely IL-1ra production was significantly enhanced by Euc-c. The expressions of IVL, CERS3 and CERS4, keratinocyte differentiation markers, were upregulated to 3.1, 2.8 and 2.7-fold respectively by Euc-c treatment, compared to the control, while the proliferation was downregulated. The lipid contents in Euc-c-treated cells tended to increase, compared with non-treated cells. To explore the underlying mechanism of these effects, we next performed siRNA experiments against PPAR-β/δ. Euc-c enhanced PPAR-β/δ mRNA expression to 3.25-fold, while PPAR-β/δ mRNA expression in transfected cells was suppressed. The expressions of IVL, CERS3 and CERS4 in transfected cells were suppressed to 1.48, 0.82 and 0.72-fold respectively, concomitant with suppression of PPAR-β/δ mRNA expression. These results indicated that Euc-c exerts anti-inflammatory effects and regulates keratinocyte differentiation via the modulation of PPAR-β/δ pathway. Therefore, the application of Euc-c is expected to exert beneficial effect on skin disorders evoked by perturbation of skin homeostasis.

Keywords: *Eucalyptus citriodora;* PPAR-β/δ, Inflammation; Barrier Function; Cutaneous Homeostasis

1. Introduction

The primary functions of the epidermis, especially stratum corneum which is comprised terminally differentiated cornified cells, are to serve permeability barrier function and water holding property. As reviewed by Menon, physical and/or structural features of stratum corneum have attracted researchers since 19th century[1]. Then the subjects of studies passed into biological and/or biochemical characteristics of keratinocytes with increasing the evidence that keratinocytes play crucial roles in cutaneous biology and immune system in 1980s[2]. To concoct these functions maturely, inter- and intracellular events undergo in sophisticated systems of different proteins and lipids during keratinocyte differentiation[3-6]. Therefore, it is important to regulate keratinocyte differentiation which is affected by skin environment such as dryness and inflammation[7-9]. Peroxisome proliferator-activated receptors (PPARs), which have been cloned as a member of the steroid hormone receptor superfamily in mouse liver, are classified three different isoforms termed PPAR-α, PPAR-β/δ and PPAR-γ sharing considerable sequence and structural homologies[10,11]. Activated-PPARs regulate the target gene expression by binding to a PPAR response element (PPRE) in the promotors of target genes as heterodimers with retinoid X receptors[12]. Initial studies demonstrated

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that PPARs are pivotal participants in the regulation of energy homeostasis by modulating glucose and lipid metabolism and transport[13],furthermore subsequent studies have shown that PPARs regulate in other cellular functions such as cell proliferation, cell differentiation, apoptosis and inflammation. The expressions of all subtypes of PPAR are identified in normal human epidermal keratinocytes. Of all the subtypes, PPAR-β/δ is expressed dominantly and consistently in keratinocytes, whereas PPAR-α and -γ are expressed lower quantities[14]. As previously reviewed the role of PPARs in skin diseases including psoriasis, atopic dermatitis and skin cancer[15-17], PPARs are involved in keratinocyte differentiation, epidermal hyperplasia, inflammation and permeability barrier function. Therefore the activation of PPAR-β/δ by exogenous ligands is expected to maintain the skin homeostasis. *Eucalyptus citriodora* (Euc-c) is an ever green tall tree, originated from temperate and tropical northeastern Australia. Their essential oil is used in perfumery and insect repellents, and applied as an alternative medicine against respiratory problems[18]. Gbenou and colleagues demonstrated that the essential oil exhibited anti-inflammation and analgesic properties in rats[19]. Further in vitro experiments showed that the essential oil and its major monoterpenes exhibited moderate to strong antioxidant activity[20], in which the constituents of the essential oil inhibited nitric oxide and prostaglandin E_2 production with altered expression of inducible nitric oxide synthase and cyclooxygenase-2[21] and that the resin inhibited the proliferation of B16F10 cells via apoptosis[22]. In this study, we demonstrated the effect of Euc-c extract on cytokine production, cell proliferation and cell differentiation in HaCaT cells, a spontaneously immortalized human keratinocyte cell line.

2. Materials and Methods

2.1 Reagents

Lipopolysaccharide (LPS) and Tumor necrosis factor-α (TNF-α) were purchased from Merk (St. Louis, MI). Ca^{2+}-free Dulbecco's modified Eagle medium (DMEM) without pyruvate and glutamine, 200mM L-glutamine, 100mM sodium pyruvate and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA).

2.2 Preparation of Euc-c extract

Leaves of Euc-c were kindly provided from Lone Pine Koala Sanctuary (Brisbane, QLD, Australia). The leaves were dried at room temperature and pulverized, followed by extraction procedure according to previous studies[23,24]. Briefly, the powder (250mg) was applied onto solid phase extraction tube and extracted with 5ml of dichloromethane and 11ml of methanol. After evaporation, the residue was dissolved in 1ml of dimethyl sulfoxide. The concentration of the solution was expressed 250mg equivalent (mge)/ml.

2.3 Cell culture

HaCaT cells, a spontaneously immortalized human keratinocyte cell line, were kindly gifted from Professor Michael Roberts at University of Queensland (Brisbane, QLD, Australia). To maintain HaCaT cells in the distinct stage of differentiation, the cells were cultured according to the method previously reported[25]. Calcium in FBS was depleted by incubation with Chelex 100 resin (Bio-Rad, Hercules, CA) for 1hr at 4°C. The resin was removed with 0.22μm filter. HaCaT cells were maintained in Ca^{2+}-free DMEM supplemented with 4mM L-glutamine. 1mM sodium pyruvate, 5% Ca^{2+}-depleted FBS, and 0.05 (LC) or 1.25 (HC) mM $CaCl_2$.

2.4 Cell proliferation assay

LC- and HC-HaCaT cells were seeded into a 96-well plate at the cell density of 2.0 x 10^3 and then were maintained in at 37°C in humidified atmosphere containing 5% CO_2 for 24hr. BrdU incorporation assay kit (Cell Signaling Technology, Danvers, MA) was employed for cell proliferation assay, according to the manufacturer's instruction.

2.5 Cytotoxicity

LC-HaCaT cells were seeded into a 96-well plate at the cell density of 2 x 10^3 cells/well and maintained in a humidified atmosphere of 5% CO_2 at 37°C for 24hr. The cells were treated with 50, 100 and 250μge/ml of Euc-c for 24hr and then subjected to neutral red assay

2.6 Cytokine production

LC-HaCaT cells were seeded into a 96-well plate at the cell density of 5 x 10^3 cells/well (for IL-6 and IL-8) or 1 x 10^4 cells/well (for IL-1ra) and maintained in a humidified atmosphere of 5% CO_2 at 37°C for 24hr. To estimate IL-6 production, the cells were treated with 100ng/ml LPS and 250μge/ml Euc-c for 24hr. For IL-8 production assay, the cells were treated with 10ng/ml TNF-α and 250μge/ml Euc-c for 24hr. The productions of IL-6 and IL-8 was measured by ELISA kit (R&D sys-

tems, Minneapolis, MN), according to the manufacturer's instruction. To measure the production of IL-1ra, the cells were treated with 250μge/ml Euc-c for 24hr. After discarding the medium, the cells were washed with PBS twice and lysed by freeze-thaw cycle three times in 200μl of PBS. After removing cell debris from the lysates by centrifugation x10,000g for 10 min, the supernatant were subjected to IL-1ra ELISA kit (R&D systems, Minneapolis, MN), according to the manufacturer's instruction.

2.7 Cell differentiation

To evaluate differentiation stage in LC- and HC-HaCaT cells, the cells were seeded into a ϕ6cm dish at 2.5 x 10^5/dish and maintained in LC-DMEM or HC-DMEM in a humidified atmosphere of 5% CO_2 at 37°C till >80% confluent, followed by real-time PCR as described below. To evaluate the effect of Euc-c on LC-HaCaT differentiation, LC-HaCaT cells maintained in LC-DMEM in a humidified atmosphere of 5% CO_2 at 37°C till >80% confluent. The cell were treated with 250μge/ml Euc-c for further 24hr and then subjected to real-time PCR.

2.8 Small interfering RNA (siRNA) Transfection

LC-HaCaT cells were seeded into a ϕ6cm dish at 2.5 x 10^5/dish and maintained in a humidified atmosphere of 5% CO_2 at 37°C for 24hr. Predesigned PPARD siRNA (NM_006238) obtained from Merk (St. Louis, MI) was transfected with Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instruction and maintained in a humidified atmosphere of 5% CO_2 at 37°C for 24hr, followed by Euc-c treatment for further 24hr. Then the harvested cells were subjected to total RNA extraction, reverse transcription and real-time PCR to assess the expression of PPAR-β/δ, IVL, CERS3 and CERS4.

2.9 Real-time PCR

Total RNA was extracted from HaCaT cells with SV RNA isolation kit (Promega, Madison, WI), according to the manufacturer's instruction and then performed reverse transcription reaction (42°C 15min, 95°C 5min) with RT system (Promega, Madison, WI). PCR amplification and detection were performed on Rotar-Gene Q (Corbett) using the initial denaturation condition of 95°C for 5 min, followed by 40 cycles at 94°C for 10sec, 62°C for 15sec and 72°C for 20sec each with primers as described in **Table 1**. Expression of target mRNA was quantified using the comparative threshold cycle (Ct) method for relative quantification ($2^{-\delta\delta Ct}$), normalized to the geometric mean of reference genes β-actin.

Name	Sequence
β-actin	forward: GATGAGATTGGCATGGCTTT reverse: CACCTTCACCGTTCCAGTTT
IVL	forward: GGCCCTCAGATCGTCTCATA reverse: CACCCTCACCCCATTAAAGA
CERS3	forward: ACATTCCACAAGGCAACCATTG reverse: CTCTTGATTCCGCCGACTCC
CERS4	forward: GGAGGCCTGTAAGATGGTCA reverse: GAGGACCAGTCGGGTGTAGA
PPARD	forward: ACTGAGTTCGCCAAGAGCAT reverse: TGCACGCCATACTTGAGAAG

Table 1. Primer sequences

2.10 Lipid accumulation in HaCaT cells

LC- and HC-HaCaT cells were seeded into a flasks (75cm^2) at 1.0 x 10^6/flask and maintained in LC-DMEM or HC-DMEM in a humidified atmosphere of 5% CO_2 at 37°C. After reaching >80% confluent, LC-HaCaT cells were treated with 250μge/ml Euc-c for 24hr, harvested by trypsinization and the cell number was adjusted to 2 x 10^6 cells/tube. After washing three times with 1ml of PBS, the cells were lyzed with 700μl of $CHCl_3$:methanol (2:1) to extract lipids followed by addition of 400μl H_2O and centrifugation at 5000g for 5min. The lower layer was transferred to glass tube and dried under vacuum.

The residue was dissolved in 20μl of $CHCl_3$:methanol (2:1). Ten μl of lipid solution was applied on a thin-layer chromatography plate and developed with $CHCl_3$:methanol:CH_3CO_2H (190:9:1) twice. After drying, the plated was charred at 180°C for 10min and subjected to dencitmetric analysis.

2.11 Statistical analysis

Data are expressed as means±SD. Statistical comparison between experimental groups and controls was performed using an unpaired Student's t-tests. P values less than 0.05 were considered significant.

3. Results

3.1 Difference in differentiation stage between HaCaT cells maintained in different concentration of Ca^{2+}

To evaluate the effect of Ca^{2+} concentration, morphological chages in LC-HaCaT cells and HC-HaCaT cells are observed. LC-HaCaT cells showed less compacted and spindle shape with absence of cell to cell tight junction (**Figure 1a**). On the other hand, HC-HaCaT cells showed a more spread-out squamous shape with tight junction among the cells (**Figure 1b**). Next, to evaluate the effect of Ca^{2+} concentration on cell proliferation, BrdU up-take assay was performed. The cell proliferation ratio was remained in HC-HaCaT cells, compared to LC-HaCaT cells (**Figure 1c**). To estimate differentiation stage, the expressions of involucrin (IVL), ceramide synthase 3 (CERS3) and ceramide synthase 4 (CERS4) in LC- and HC-HaCaT cells were evaluated. The expressions of IVL was enhanced 3.6-fold with significance in HC-HaCaT cells, compared with LC-HaCaT cells. Likely, CERS3 and CERS4, which are dominantly expressed in differentiated keratinocytes, were expressed significantly in higher levels in HC-HaCaT cells, compared with LC-HaCaT cells (**Figure 1d**).

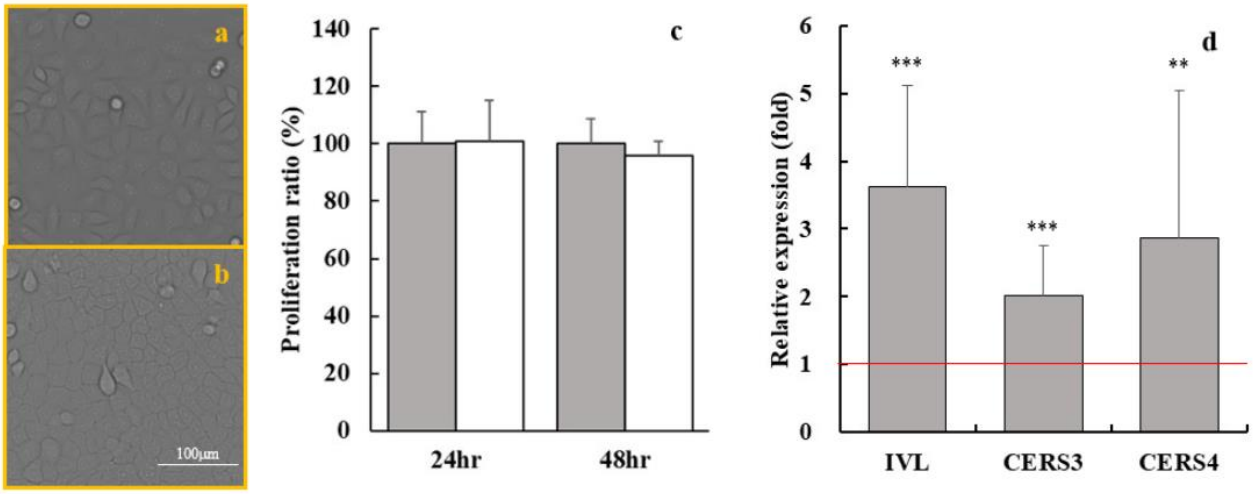

Figure 1; Differences on morphology, cell proliferation and differentiation maker expression between LC-HaCaT and HC-HaCaT. Less compacted and spidle shape with absence of tight junction was observed in LC-HaCaT (a), while HC-HaCaT cells showed spread-out squamous shape with tight junction (b). There was no significant difference in cell proliferation ratio (c) between LC-HaCaT (closed column) and HC-HaCaT cells (open column). Significant enhancement of differentiation marker expression was detected in HC-HaCaT cells, compared with LC-HaCaT cells (d). Each value represents the mean ± SD from three independent experiments (**p<0.01, ***p<0.001 compared with LC-HaCaT).

3.2 Cytotoxicity of Euc-c

The cytotoxicity of Euc-c on LC-HaCaT cells was evaluated with neutral red assay to determine the concentration of Euc-c for further experiments. Significant cytotoxicity was not detected within the range of concentration applied in this experiment (**Figure 2**). Therefore, the cells were treated with 250μge/ml in the following experiments.

3.3 Anti-inflammatory effect of Euc-c

To evaluate the anti-inflammatory effect of Euc-c, the production of IL-6, IL-8 and IL-1ra from LC-HaCaT cells was measured with ELISA. LPS-stimulation induced 904±38pg/ml of IL-6 secretion while unstimulated LC-HaCaT cells secreted 31.1±4.0pg/ml. The enhanced production of IL-6 was significantly reduced to 653±50pg/ml in the cells treated with Euc-c (**Figure 3a**).

IL-8 production was enhanced to 251±51pg/ml with stimulation of TNF-α, while the production in untreated LC-HaCaT cells was 23.8±0.5pg/ml. The enhanced production of IL-8 was significantly suppressed to 80.5±6.6pg/ml in the cells treated with Euc-c (**Figure 3b**). IL-1ra production in Euc-c-treated cells was significantly enhanced to 1371±93pg/ml, compared with the untreated cells (**Figure 3c**).

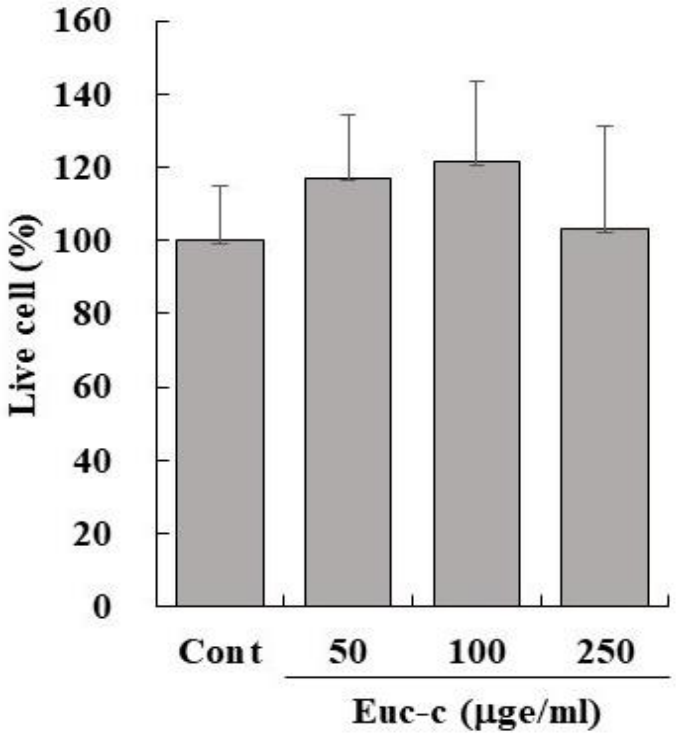

Figure 2; Cytotoxicity of Euc-c. Significant cytotoxicity was not detected at range of concentration applied in this experiment. Each value represents the mean ± SD from three independent experiments.

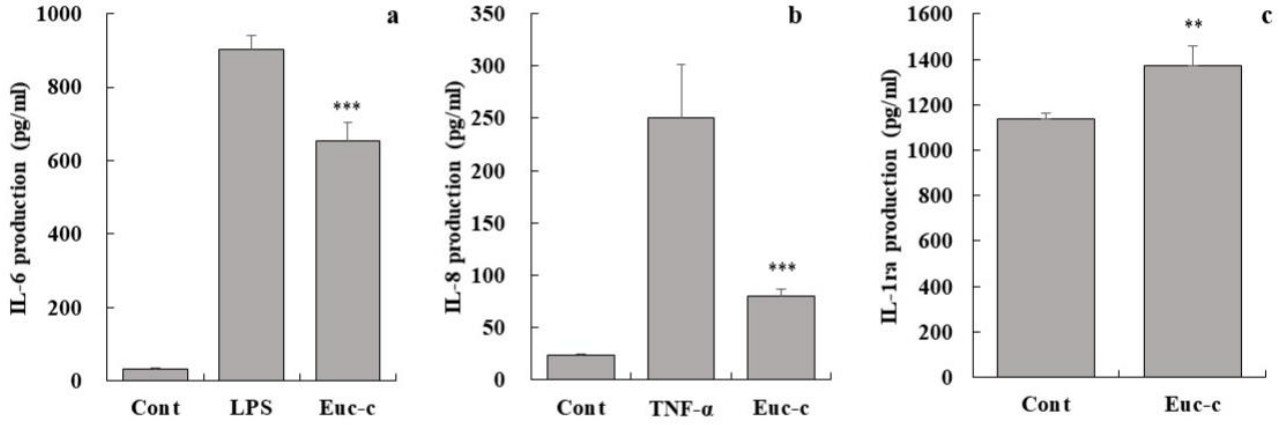

Figure 3; Anti-inflammatory effect of Euc-c. To evaluate the anti-inflammatory effect of Euc-c, the production of IL-6 (a), IL-8 (b) and IL-1ra (c) from LC-HaCaT cells was measured. LPS-induced IL-6 production was significantly reduced (a). TNF-α-induced IL-8 was also reduced significantly (b). IL-1ra production in Euc-c-treated cells was significantly enhanced (c). Each value represents the mean ± SD from three independent experiments (**p<0.01, ***p<0.001 compared with the production from stimulated cells)

3.4 Euc-c suppresses proliferation of LC-HaCaT

BrdU up-take assay was employed to estimate whether Euc-c has effects on LC-HaCaT proliferation ratio. The relative proliferation of LC-HaCaT cells treated with Euc-c was decelerated to 33.0±2.4% of the

control with significance (**Figure 4**).

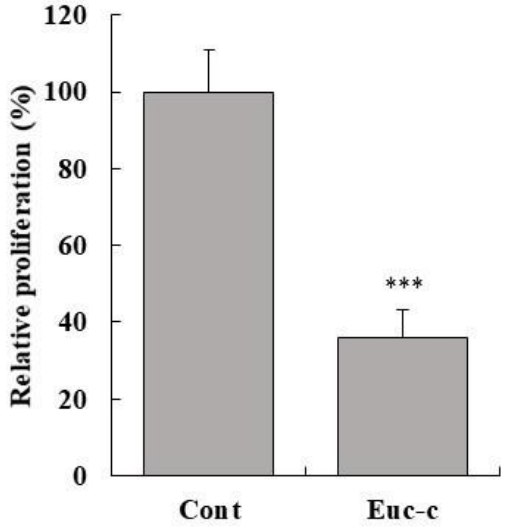

Figure 4; Euc-c suppresses proliferation ratio of LC-HaCaT. The relative proliferation of LC-HaCaT cells treated with Euc-c was significantly decelerated. Each value represents the mean ± SD from three independent experiments (***p<0.001 compared with the control)**.**

3.5 Euc-c promotes LC-HaCaT differentiation

To examin the effect of Euc-c on LC-HaCaT differentiation, the relative expressions of IVL, CERS3 and CERS4 were assessed. As shown in **Figure 5**, every expression of IVL, CERS3 and CERS4 was significantly accelerated to 3.1, 2.8 and 2.7-fold respectively, compared to the control.

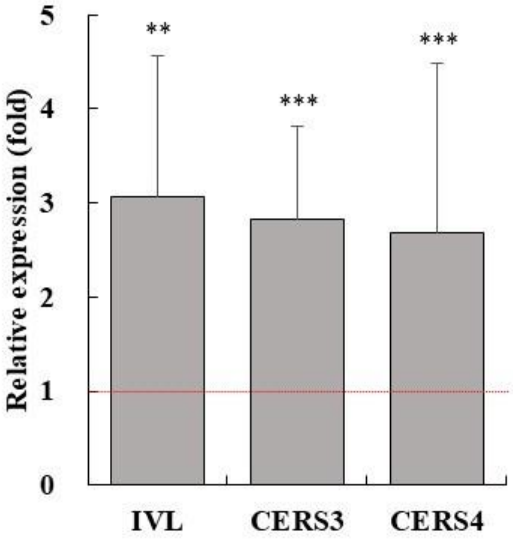

Figure 5; Euc-c promotes LC-HaCaT differentiation. The expressions of IVL, CERS3 and CERS4 was significantly accelerated. Each value represents the mean ± SD from three independent experiments (**p<0.01, ***p<0.001 compared with the control)**.**

3.6 Euc-c enhances lipid accumulation in HaCaT cells

Because lipid accumulation in keratinocytes is important to exert skin barrier function, we examined whether Euc-c is involved in lipid accumulation in LC-HaCaT cells. The upper spots were detected in all lipid extract from LC-HaCaT cells, Euc-c-treated LC-HaCaT cells and HC-HaCaT cells (**Figure 6**). The results in dencitmetric analysis showed an increase in the intensity of the upper spot in Euc-c-treated LC-HaCaT cells, compared to LC-HaCaT cells and HC-HaCaT cells (**Table 2**). On the other hand, the lower spot was detected in Euc-c-treated LC-HaCaT cells, while the spot was not detected in LC-HaCaT cells and HC-HaCaT cells

(**Figure 6**).

	Upper spot	Lower spot
LC-HaCaT	1539000	ND
Euc-c	1664000	791400
HC-HaCaT	1543000	ND

Table 2. Dencitmetric analysis of lipid spots

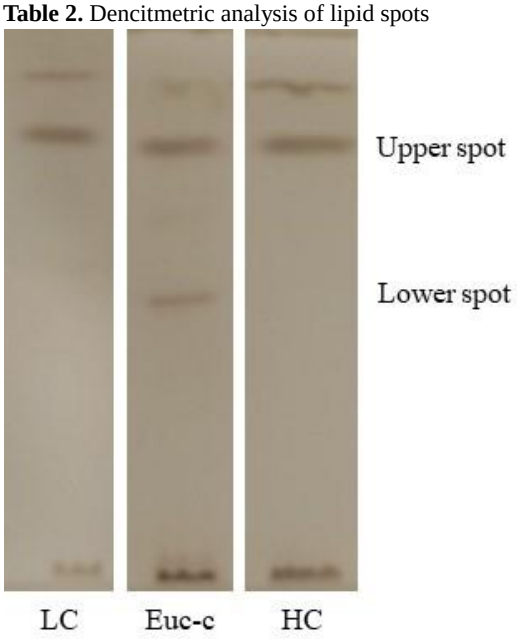

Figure 6; Euc-c enhances lipid accumulation in HaCaT cells. The upper spots were detected in all lipid extract from LC-HaCaT cells, Euc-c-treated LC-HaCaT cells and HC-HaCaT cells. The lower spot emerged only in Euc-c-treated LC-HaCaT cells**.**

3.7 PPAR-β/δ is involved in the effects induced by Euc-c

Since the results described above strongly suggested that Euc-c exerts the effects on LC-HaCaT cells through PPAR-β/δ activation, we performed a siRNA-mediated PPAR-β/δ knockdown in LC-HaCaT cells to examine whether PPAR-β/δ might be activated by Euc-c. PPAR-β/δ siRNA reduced the PPAR-β/δ mRNA level in Euc-c-treated LC-HaCaT cells by 0.56 fold compared with the control, while Euc-c treatment enhanced PPAR-β/δ expression to 3.25-fold in siRNA-untransfected LC-HaCaT cells (**Figure 7a**). The mRNA expression levels of IVL, CERS3 and CERS4 in siRNA-transfected LC-HaCaT cells treated with Euc-c were significantly reduced to 1.48, 0.82 and 0.72-fold respectively, whereas Euc-c treatment enhanced the expression levels in wild-type LC-HaCaT cells (**Figure 7b**).

4. Discussion

The primary function of the epidermis, which is located in the outmost of the body, is to serve a permeability barrier between the external environment and the host. In order to provide the mature permeability barrier, the orchestrated inter- and intracellular events undergo in the epidermis. Due to the disturbance of skin micro-environment including inflammation, cell proliferation and cell differentiation impairs the permeability barrier function[7-9], it is important to regulate complex biological events in the epidermis. Since PPARs are initially identified as transcriptional regulators of lipid and glucose metabolism[10,13], further evidence has also

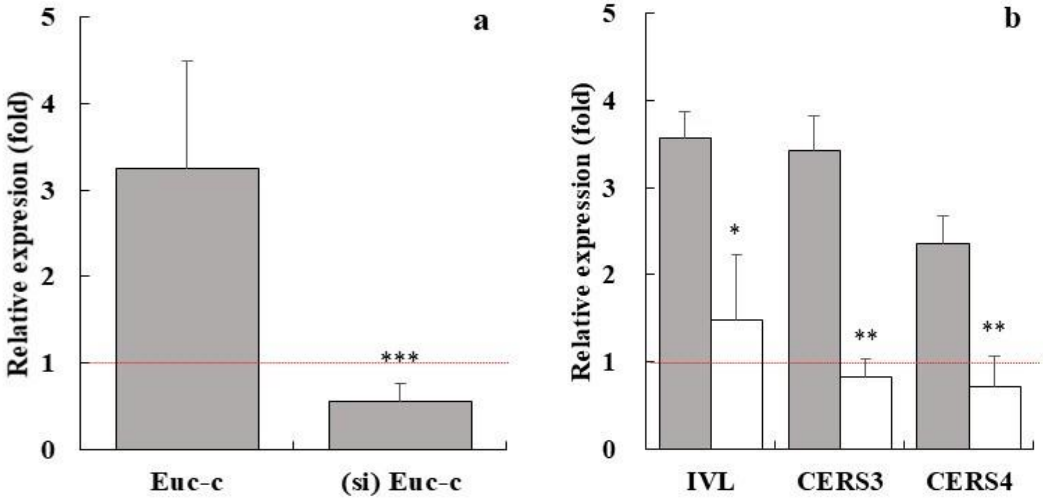

Figure 7; PPAR-β/δ is involved in the effects induced by Euc-c. PPAR-β/δ siRNA reduced the PPAR-β/δ mRNA level in Euc-c-treated LC-HaCaT cells compared with Euc-c-treatet cells (a). The mRNA expression levels of IVL, CERS3 and CERS4 in siRNA-transfected LC-HaCaT cells treated with Euc-c (open column) were reduced significantly (b). Each value represents the mean ± SD from three independent experiments (*p<0.05, **p<0.01, ***p<0.001 compared with the untransfected cells).

accumulated for their tissue distribution and other functions. Regarding their distribution in human skin, previous studies showed that all PPAR isotypes are expressed in human skin and that PPAR-β/δ is ubiquitously expressed throughout the epidermis while the expressions of PPAR-α and -γ increase along with the keratinocyte differentiation[14,26]. In addition, other studies on their function in the skin demonstrated that PPARs were involved in keratinocyte differentiation, epidermal hyperplasia, inflammation and permeability barrier function[15-17]. Euc-c is an ever green tall tree, originated from temperate and tropical northeastern Australia. The diverse biological activities of Euc-c extract, especially as an essential oil, have been reported previously[18-22] and its safety has been proved by long history of traditional usage. In this study, we demonstrated the effect of Euc-c extract on cytokine production, cell proliferation and cell differentiation in HaCaT cells. To elucidate the diverse events involved in inflammation, cell proliferation and differentiation in epidermis, normal human epidermal keratinocytes (NHEKs), which are supplied commercially, provide an ideal in vitro experimental system. However their usage is limited due to the complexities involved in cultivation and a limited number of passages. HaCaT cells, which have been established as spontaneously immortalized human keratinocytes maintaining full epidermal differentiation capacity[27], are widely employed as a model for the study of keratinocyte functions to exclude the problems arose in the system using NHEKs. Similar to NHEKs, Ca^{2+} addition induces morphological changes, concomitant with expressions of differentiation makers such as keratin 1, keratin 10 and involucrin in HaCaT cells[28]. Interestingly, Deyrieux *et al*. reported that HaCaT cells exhibited reversible differentiation by switching the Ca^{2+} concentration from 2.8mM to 0.03mM[25]. First, we elucidated the differences in morphology, proliferation rate and differentiation maker expression between LC- and HC-HaCaT cells. As showed in **Figure 1,** LC-HaCaT cells exhibited less compacted spindle shape with less mRNA expression of IVL, CES3 and CERS4, whereas HC-HaCaT cells exhibited a more spread-out squamous shape with enhanced mRNA expressions of the markers and maintained proliferation rate. These results suggested that HaCaT cells cultured in low Ca^{2+} condition maintained a basal-like state both morphologically and biochemically, and that the differentiated-state was induced and maintained, once the cells were cultured in high Ca^{2+} condition. We employed LC-HaCaT cells for further experiments, while HC-HaCaT cell were used as the differentiated-state control. The evidences for anti-inflammation effects of PPARs activators have been examined previously[29], therefore, the effect of Euc-c on cytokine production at a concentration of 250μge/ml, which was the highest no-cytotoxic concentration, was examined. Euc-c treatment down-regulated LPS-induced

IL-6 production and TNF-α-induced IL-8 production (**Figure 3a** and **b**). The initial demonstration of a regulatory function of PPAR-α in inflammation signaling was obtained in PPAR-α-deficient mice that displayed an exacerbated response to inflammatory stimuli[30]. Consequently, intensive studies on the effects of PPAR activators on inflammatory responses showed that all of PPAR isotypes exerted distinct and overlapping anti-inflammatory effects, as listed in **Tables 3**[31-39]. Regarding the underlying mechanism of the inhibition of pro-inflammatory cytokines, Ricote and Glass proposed five diversity mechanisms of PPAR-mediated transrepression; i) direct interaction, ii) induction of IκBα, iii) regulation of kinase activity, iv) coactivator competition and v) co-repressor interaction[40].

Up-regulation	Down-regulation
IL-4, IL-5, sIL-1ra	IL-1β, IL-6, IL-12, IL-23, IL-27
	CCL2 (MCP-1), CCL4 (MIP), CXCL8 (IL-8)
	IFN-γ, TNF-α
	ICAM-1, VCAM-1
	ET-1
	COX-2, iNOS

Table 3. Influence of PPAR activation on inflammatory molecule expression

Since NF-κB is a crucial transcriptional factor in both LPS and TNF-α signaling cascade, NF-κB may be involved in the underlying mechanism of the down-regulation of IL-6 and IL-8 by Euc-c. In contrast, IL-1ra production was up-regulated by Euc-c (**Figure 3c**). Previous reports showed that WY14643, a PPAR-α agonist, and GW501516, a PPAR-β/δ agonist, enhanced IL-1ra production and that IL-1ra gene was a direct target of PPARs[41,42]. Our results, which are consistent with previous studies, suggest that Euc-c exerts an anti-inflammatory effect by both down-regulating expression of pro-inflammatory genes and direct up-regulation of anti-inflammatory gene through PPAR activation. The function of PPAR-β/δ in keratinocyte proliferation still remains controversial. Some studies showed that PPAR-β/δ accelerates keratinocyte proliferation in psoriasis[43] and that PPAR-β/δ played a vital role in EGF-stimulated proliferation of HaCaT cells[44]. Conversely, other reports demonstrated that activation of PPAR-β/δ by GW0742 inhibited proliferation and enhanced terminal differentiation in keratinocyte[45], and that GW0742 inhibited cell growth of human N/TERT-1 keratinocytes[46]. We also obtained the contradictory results on cell proliferation by the different methods, neutral red assay and BrdU cell proliferation assay. The result in neutral red assay showed that Euc-c treatment did not affect cell viability whereas the proliferation of HaCaT cells treated with Euc-c was significantly reduced in BrdU cell proliferation assay, as well as GW501516, a selective PPAR-β/δ activator (data not shown). Because BrdU cell proliferation assay reflects BrdU incorporation during DNA synthesis, our results suggest that the ratio of cells in S phase was decreased by Euc-c treatment. However, further experiments are required to elucidate the effect of Euc-c on cell cycle. Next, we evaluated the expression of keratinocyte differentiation markers in Euc-c-treated LC-HaCaT cells. The mRNA expression of IVL, a well-known keratinocyte differentiation marker, was accelerated in the cells treated with Euc-c. Concurrently, the mRNA expressions of CERS3 and CERS4 were enhanced by Euc-c treatment (**Figure 5**). The enzymes are dominantly expressed in keratinocyte[47] and their expressions are upregulated upon keratinocyte differentiation[48]. Previous studies demonstrated that the activation of PPARs induced keratinocyte differentiation[49,50]. Westergaard and the colleagues showed that L165041, a selective PPAR-β/δ activator, was the most potent for keratinocyte differentiation, compared with PPAR-α and PPAR-γ activators[26]. The results we obtained also suggest that Euc-c treatment induced keratinocyte differentiation. Lipids including ceramide, cholesterol and free fatty acid are required to establish a mature permeability barrier. The deficiency of essential fatty acids, which are components of ceramide, results in abnormalities of permeability barrier[51]. Moreover, previous studies showed that PPAR-β/δ activators improved

epidermal barrier homeostasis through stimulation of lipid synthesis[52,53]. We showed here that the lipid extract from Euc-c-treated LC-HaCaT cells demonstrated two lipid spots (the upper and the lower spot) whereas the extract from LC-HaCaT and HC-HaCaT cells exhibited only the upper spot (**Figure 6**). Dencitmetric analysis revealed that the treatment of Euc-c tended to increase the intensity of the upper spot, compared to LC- and HC-HaCaT cells (**Table 2**). Scince the lower spot was detected in the extract of LC-HaCaT cells treated with GW501516 as well (data not shown), the spot was not a result of Euc-c extract. Thus the emergence of the lower spot suggests that the treatment with Euc-c induced synthesis of another kind of lipid. Because the upregulation of CERS3 and CERS4 mRNA expression was found, and Chon *et al*. demonstrated that an oat lipid extract enhanced ceramide synthesis via PPAR pathways[54], the enhancement of ceramide synthesis can be presumed although ceramide spots could not be detected in our experimental conditions. Finally, we performed siRNA experiments for PPAR-β/δ to clarify the effects of PPAR-β/δ on the above effects of Euc-c. According to results described above, we at first hypothesized that Euc-c activated PPAR-β/δ pathway. However, the treatment with Euc-c accelerated the expression of PPAR-β/δ mRNA expression which was suppressed by siRNA transfection (**Figure 7a**). Concomitantly, the mRNA expressions of IVL, CERS3 and CERS4, which were upregulated by Euc-c, were suppressed by siRNA transfection (**Figure 7b**). It is still unclear whether Euc-c activates PPAR-β/δ, however we confirmed here that Euc-c upregulated the mRNA expression of PPAR-β/δ, accompanied by enhancement of the accumulation of fatty acids which can be endogenous ligands for PPAR-β/δ. Taken together, these results suggest that Euc-c affected keratinocyte functions via modulation of PPAR-β/δ pathway. Atopic dermatitis is a chronic and relapsing disease characterized skin barrier dysfunction and immune dysregulation. The application of Euc-c is expected to exert beneficial effect on atopic dermatitis by improving both skin barrier dysfunction and immune dysregulation, as well as other PPAR-β/δ activators (Panduratin A and GW0742) previously reported[55,56]. On the other hand, because previous studies demonstrated that the modulation of PPAR-β/δ pathway exhibited therapeutic effects on wound healing[57], melanoma[58, 59] and UV-induced extrinsic skin aging[60], further experiments evaluating the effects of Euc-c on other skin disorders should be explore to develop the application of Euc-c for a wide range of skin disorders.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript

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