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갈로카테킨-3-갈레이트가 풍부한 열전환 카테킨의 피부 장벽 회복에 대한 개선 효과

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Effect of Heat-epimerized-catechin-mixture Rich in Gallocatechin-3-gallate on Skin Barrier Recovery

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요 약: 지금까지 (-)-epigallocatechin-3-gallate (EGCG)는 인간의 피부에 유용한 녹차 카테킨 중에서 가장 강력한 항산화 성분으로 알려져 왔다. 본 연구팀은 용매, 온도, 압력 등 다양한 조건을 변화시키며, 멸균과정(autoclaving) 중에 발생하는 이성질체화(epimerization) 과정을 연구하여, gallocatechin-3-gallate (GCG) 함량이 크게 증가된 열전환-EGCG-복합체(heat-epimerzied-EGCG-mixture, HE-EGCG-mix)를 순수한 EGCG로부터 조제 하였다. 이러한 열전환-EGCG-복합체는 무모쥐 SKH-1을 이용한 실험에서, 손상된 피부 장벽의 회복 시에 인보루크린 7 (involucrin 7) 단백질의 발현량을 EGCG 처리 시보다 증가시킴을 확인하였다. 또한, *in vitro* 실험을 통하여 GCG는 PPAR-α에 대한 전이활성(transactivation) 효과가 EGCG보다 뛰어남을 확인하였다. 이러한 결과는 열전환-EGCG-복합체에 함유된 고함량의 GCG 성분에 의해서, 피부 장벽 손상 회복 시 PPAR에 의해 매개된 각질형성세포(keratinocyte)의 분화가 더욱 촉진될 수 있음을 암시한다. 따라서, EGCG의 C-2 에피머(epimer)인 GCG는 녹차 카테킨을 이용한 피부 장벽 개선 용도의 화장품과 건강식품 개발 시 주요 소재로 활용될 수 있다.

Abstract: Until now, (-)-epigallocatechin-3-gallate (EGCG) is known as the most powerful antioxidant among green tea catechins having many beneficial effects on human skin. Considering that the content of catechins is variable according to many conditions such as solvent, temperature and pressure, we prepared the heat-epimerized-EGCG-mixture (HE-EGCG-mix) containing high content of gallocatechin-3-gallate (GCG) by epimerization during autoclaving process and found out its optimal condition for maximizing conversion from EGCG to GCG. To investigate the effects of EGCG and HE-EGCG-mix on skin barrier function, we performed *in vivo* experiments with hairless mice. We found that HE-EGCG-mix has more potent stimulating activity than EGCG for the production of involucrin 7 (INV7) and for recovery of barrier function in SKH-1 mice. Also, we found that GCG stimulates PPAR-α transactivation more effectively than EGCG *in vitro* by transient transfection assay for PPAR-α activation activity. These imply that HE-EGCG-mix consisting of high content of GCG should stimulate more efficiently recovery of skin barrier through PPAR-mediated-kerationocyte differentiation than EGCG. In conclusion, our study may provide a possibility that GCG, the C-2 epimer of EGCG, could be a potentially effective agent for development of new cosmetics or health foods for recovery of skin barrier.

Keywords: *epimerization, gallocatechin-3-gallate, involucrin, skin barrier function, PPAR-α.*

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1. Introduction

The 4 major polyphenolic catechins present in green tea leaves are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epigallocatechin-3-gallate (EGCG), which is the most abundant. The total polyphenols are readily extracted from green tea leaves by water or organic solvents such as methanol and ethanol, so their contents are variable by extraction condition such as solvent, temperature and pressure[1].

Many previous studies showed that flavanol including catechins can be epimerized to its epimer under certain conditions such as the autoclave process during the production of green tea beverages[2]. This epimerization reaction may be the key role in the changes of sensory qualities, contents of each ingredient, and actual effects of processed green tea beverages[2]. For example, GCG, the C-2 epimer (2*S*, 3*R*) of the original tea catechin EGCG, might be more effective than EGCG in producing a precipitate with cholesterol in micelles[3] and in inhibiting mouse type IV allergy[4] and MMP-9 activity[5].

There have been more than 150 reports of *in vivo* and *in vitro* studies on the effects of green tea on the skin[6]. EGCG is the most powerful antioxidant among green tea catechins and has many beneficial effects on human skin. The influence of EGCG on MMP expression and activity in UV radiation-damaged dermal fibroblast cells was reported recently[6,7]. In addition, it was found that EGCG activates the coordinated expression of p57 and caspase 14 in normal human epidermal keratinocytes (NHEKs), which facilitates terminal differentiation of these cells and is related to the recovery of skin barrier function[8]. It also has been shown that oral administration of green tea in mice not only inhibits skin tumorigenesis[9] but also reduces fatty tissues in the dermis[10].

The primary function of the skin epidermis is to produce the protective, semi-permeable stratum corneum that permits terrestrial life[11]. The barrier function of the stratum corneum is provided by patterned lipid lamellae localized to the extracellular spaces between corneocytes. The regulation of barrier lipid synthesis has been studied in a variety of models and the induction of several enzymes or proteins has been demonstrated during keratinocyte differentiation. For example, the transition from the spinous to the granular layer is accompanied by up-regulation of genes such as filaggrin, involucrin, and transglutaminase[12]. In addition, peroxisome proliferator-activated receptor (PPAR)-α stimulates differentiation and apoptosis and decreases proliferation in cultured human keratinocytes and *in vivo* when applied topically to mouse skin[13,14]. Therefore, agents that activate PPAR-α and up-regulate the expression level of specific marker genes of keratinocyte differentiation in epidermis can be used to fortify skin barrier function and protect human skin from harmful environments.

However, no studies have been done regarding epimerized catechins may cause stimulatory effect on skin barrier recovery. In this study, we prepared the heat-epimerized-EGCG-mixture (HE-EGCG-mix) rich in GCG by epimerization during autoclaving process and examined that HE-EGCG-mix stimulates skin barrier recovery, involucrin expression in epidermis *in vivo*. Through these experiments, we found out the optimal condition for maximizing conversion to GCG from EGCG to prepare HE-EGCG-mix and concluded that GCG contained above 50 % in the HE-EGCG-mix might be PPAR-α ligand and its stimulative effect on skin barrier recovery.

2. Materials and Methods

2.1. Samples and Cell Culture

All reagents and solvents for activity tests were purchased from commercial suppliers (Sigma-Aldrich, Fluka, USA) and used without further purification. All other chemicals of analytical grade were purchased from Merck (USA). Cell culture reagents were purchased from Invitrogen, Cambrex, Gibco, and Welgene. CV-1 cells (ATCC CCL70, VA, USA) were grown under the same conditions as above except without phenol red. For transient transfection, the medium was exchanged with medium supplemented with 10 %

charcoal-stripped fetal bovine serum 4 h before the experiment.

2.2. Preparation of Heat-epimerized-EGCG-mixture Rich in Gallocatechin-3-gallate

EGCG from green tea was purchased from commercial suppliers (Teavigo, DSM, USA) and used without further purification. Pure EGCG (4 g) was dissolved in 10 mL of the BG solvent (3 : 7 = butylene glycol : 0.1 M citric acid buffer with pH 5 ~ 6.5). The solution was then autoclaved for 30 min at 120 ℃. The changes in total EGCG and formation of GCG were monitored using HPLC as described below. The samples were analyzed using a HPLC system (Waters 2695) connected to a UV-Vis detector (Waters 2996 photodiode array detector) set at 280 nm. Identification and quantification of two catechins were achieved by comparing retention times and peak areas on the chromatograms with the references.

2.3. Animals and Skin Barrier Disruption

Six to eight-week old female hairless mice (SKH-1, Samtako Co., Korea) were used in this study. Age matched mice from the same genetic background were used as controls. The animal procedures were approved by the local Animal Studies Subcommittee and performed in accordance with their guidelines. The epidermal barrier was disrupted in SKH-1 hairless mice by repeated applications of absolute acetone to the dorsal region for approximately 30 s. Immediately after breaking the barrier (TEWL rates exceeding 4.0 mg/cm^2/h), appropriate concentrations of catechins solubilized in propylene glycol : ethanol (7 : 3 v/v) or the propylene glycol : ethanol vehicle alone (200 μL total volume) were applied topically to a 5 cm^2 area of acetone-treated skin twice a day for 3 days. For recovery calculation, TEWL was determined at before disruption, 0, 3, 6, and 24 h following disruption. After disruption 3 days, skin biopsy was performed to analysis of Western blot and immunostaining. (Recovery (%) = (TEWL immediately after barrier disruption - TEWL at indicated time point) / (TEWL immediately after barrier disruption - baseline TEWL) × 100)

2.4. Western Blot and Immunohistochemistry

The epidermis was collected by trypsinization (0.25 % trypsin with Na_4EDTA Gibco, Invitrogen, USA) of the skin from an area of about 6 mm diameter on the dorsal region of hairless mice. A protein lysate was prepared with cell lysis buffer (Cell Signaling Co., USA) and the proteins were quantified with a BCATM Protein Assay (PIERCE, Rockford, IL, USA). Equal amounts of protein were electrophoresed on NuPAGE® Novex Bis-Tris-Gel (Invitrogen, USA) and transferred to a nitrocellulose membrane (LC2001 Invitrogen, USA). Transglutaminase 1 (TG1) was detected with a goat polyclonal antibody (1 : 1000 in 0.1 % Tween 20 and nonfat milk) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-goat IgG-HRP (Zymed). Involucrin 7 (INV7) was detected with a rabbit polyclonal anti-mouse INV7 antibody (COVANCE, Berkeley, CA, USA) (1 : 1000 in 0.1 % Tween 20 and nonfat milk) and anti-rabbit IgG-HRP (Amersham). Actin was detected with a rabbit anti-actin antibody (Sigma, USA). Blotting proteins were visualized by enhanced chemiluminescence (Amersham, Buchinghamshire, UK). Image analysis was performed using Image J version 1.34 (NIH, USA).

Immunohistochemistry was performed as described by Lee *et al.*[15]. Tissues were fixed in 3.7 % *p*-formaldehyde and embedded in paraffin and 4 ~ 6 μm thick sections were prepared. Monoclonal antibodies to INV7 (COVANCE, CA, USA) were diluted 1 : 250 and incubated overnight. Image analysis was performed using Image Pro Plus version 4.5 (Media Cybernetics, Silver Spring, MD, USA). Each measurement was evaluated under constant magnification. For each frame, the tracing was repeated three times and expressed as the mean.

2.5. Transient Transfection Assay for PPAR-α Activation Activity

Transfection of CV-1 cells was performed with a TransFast Transient transfection kit (Promega, USA). Briefly, a unique co-precipitate containing luciferase reporter plasmids with the PPAR-α ligand-binding site and the PPAR-responsive element (PPRE) plus the

Renilla luciferase plasmid (pRL-SV40) was prepared and aliquoted into different wells to ensure that all samples were transfected with the same amount of plasmid DNA (330 ng of the luciferase reporter plasmid plus 5 ng of the Renilla luciferase plasmid per well). After 24 h at 37 ℃, the CV-1 cells were washed and incubated for 24 h in media containing the tested catechins (10 μM, 100 μM) in 0.1 % DMSO. Luciferase assays were performed using a luminometer (Lumat 9501, Berthold, Germany) with a Dual-Luciferase Reporter Assay System Kit (Promega, USA) following the manufacturer's instructions. Luciferase activities were normalized to the Renilla luciferase activities. The normalized luciferase activities versus the controls were expressed as the mean ± S.D. values of triplicate samples. Each experiment was repeated at least twice.

2.6. Statistical Analysis

Statistical significance was determined by the Student's *t*-test (TEWL assay and promoter activity assay) and the Wilcoxon *t*-test (immunohistochemistry). p values of 0.05 were considered to be statistically significant.

3. Results and Discussion

3.1. Preparation of HE-EGCG-mix Rich in Gallocatechin-3-gallate

Effect of pH on formation of GCG was examined in the present study. As a result, the formation of GCG from EGCG was pH-sensitive (Table 1). The formation of GCG was most efficient at pH 6.0 when EGCG dissolved in the BG solvent (3 : 7 = butylene glycol : 0.1 M citric acid buffer) was autoclaved at 120 ℃ for 30 min. However, the observed pH-dependent efficiency to formation of GCG was somewhat in disagreement with the reports by Chen *et al.*[1]. These finding suggested that formation of a certain useful catechin or its mixture is easily accomplished by heat-epimerization process varying the solvent system, pH and so on. But, this also suggested that other ingredients, as is citric acid and ascorbic acid, used in production of commercially available soft drinks might interact with catechins and exhibit varying catechin contents during autoclaving process for food sterilization. In final preparation with 100 mL reaction at that condition, the HPLC analysis found that HE-EGCG-mix contained (%) : EGCG 45.0 ± 0.35 and GCG 54.0 ± 0.21.

Table 1. Effect of pH on the Formation of Gallocatechin-3-gallate (GCG)

pH	GCG content (%)
5.0	52.3 ± 0.1
5.5	53.9 ± 0.6
6.0	55.5 ± 0.6**
6.5	54.7 ± 1.7

When epigallocatechin-3-gallate (EGCG) was autoclaved at 120 ℃ for 30 min, the pH of solution on the formation of GCG was very important. Data are expressed as mean ± S.D. of n = 4 samples **$p < 0.01$.

3.2. HE-EGCG-mix Stimulates Skin Barrier Recovery

Since the vital function of epidermis is to provide a barrier to transepidermal water movement, we determined whether topically applied HE-EGCG-mix enhances the recovery of epidermal permeability barrier[16]. The barrier of adult female hairless was disrupted as followed by treatment with HE-EGCG-mix. Interestingly, this mixture enhanced the recovery rates of the epidermal permeability barrier function more than EGCG did (Figure 1).

3.3. HE-EGCG-mix Increases Involucrin 7 in Hairless Mice

We next compared the changes in the expression of thestructural protein involucrin 7 (INV7) and its related-enzyme transglutaminase 1 (TG1) following topical treatment with 1.0 % (w/v) EGCG or HE-EGCG-mix, with oleanolic acid as the reference. These proteins are localized to the spinous/granular layers in the epidermis[17,18]. However the TG1 protein level did not increase after topical application, the marker protein INV7 at the late stage of keratinocyte differentiation was markedly increased in the epidermis treated with HE-EGCG-mix as compared with epidermis treated with

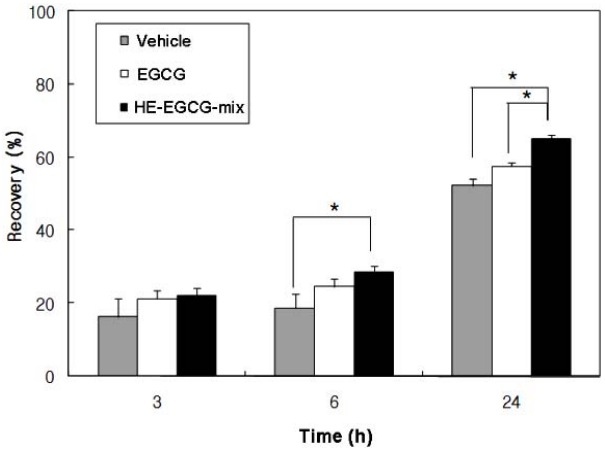

Figure 1. The skin barrier recovery effect of HE-EGCG-mix (1.0 %) on trans-epidermal water loss (TEWL). Epidermal hyperproliferation was produced by repeated barrier disruption in adult hairless mice. The animals were then treated twice a day for 3 days with the vehicle alone, EGCG or HE-EGCG-mix. $^{*}p < 0.05$, $^{**}p < 0.01$.

EGCG, regardless of the vehicle used (data not shown).

Quantitative analysis using computational image-analysis software demonstrated that when HE-EGCG-mix was applied to the hairless mouse skin, the amount of INV7 was increased about 4-fold over that of the negative control, whereas EGCG increased the amount of INV7 about only 2-fold (Figure 2).

These results suggest that HE-EGCG-mix has more potent stimulating activity than EGCG for the production of INV7 and for recovery of barrier function in SKH-1 mice. Whether this is due to better permeation through cell membranes, or to a specific adaptability of this molecule to interact with the molecular target, still remains to be elucidated. However TG1 protein, another marker of keratinocyte differentiation, did not increase after topical application to mouse epidermis. This result implies that HE-EGCG-mix affects INV7, but not TG1, during mouse epidermal differentiation.

3.4. Effect of Two Catechins on Expression of PPAR-α

We investigated the trans-activational effects of catechins on the PPRE using a luciferase reporter gene construct, 5′-GATCCCCGAACGTGACCTTTGTCCTGGTCC-3′, co-transfected with human PPAR-α

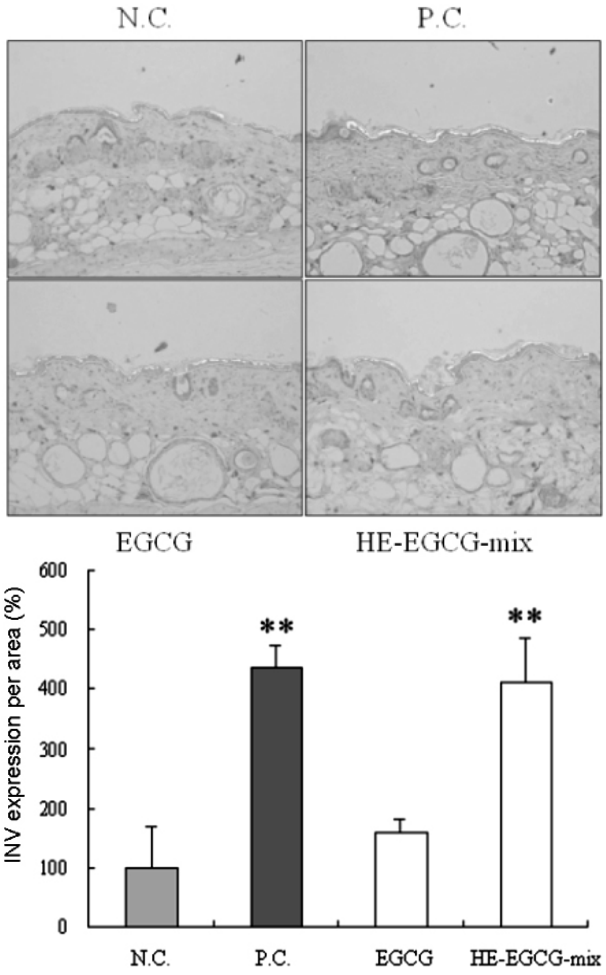

Figure 2. Effect of topical HE-EGCG-mix (1.0 %) treatment on the localization of involucrin in hyperproliferative skin. Epidermal hyperproliferation was produced by repeated barrier disruption in adult hairless mice. The animals were then treated twice a day for 3 days with the vehicle alone, 1 % oleanolic acid, EGCG or HE-EGCG-mix. The staining area of INV (yellow) was visualized by immunolocalization and analyzed per total epidermal area (green). N.C., negative control, vehicle alone P.C., 1 % oleanolic acid positive control; EGCG, epigallocatechin gallate; HE-EGCG-mix, heat-epimerized-EGCG-mixture; $^{**}p < 0.01$, $^{***}p < 0.001$.

in CV-1 cells. Expression of the endogenous gene and the reporter construct was activated in a ligand-dependent manner by PPAR. There was no effect on cell viability in CV-1 cell lines treated with up to 200 μM catechin for 24 h. Although EGCG and GCG have similar structures, GCG stimulated PPAR-α activation

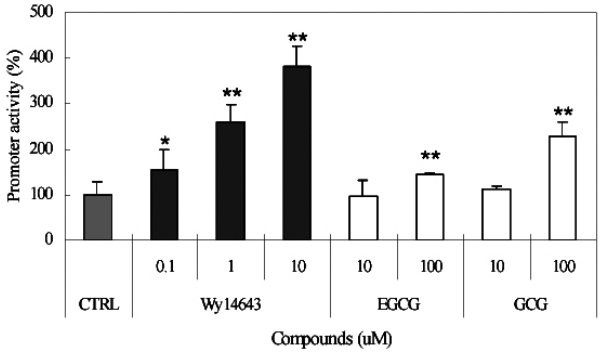

Figure 3. Effect of two catechins on PPAR-α dependent PPRE promoter activity. Wy14643 (0.1, 1, 10 μM) was used as the reference compound. Results on PPAR-dependent PPRE promoter activity are expressed as the percent of normalized luciferase activity versus the control and represent the means ± S.D. of three experiments performed in triplicate. CTRL, control; EGCG, epigallocatechin gallate; GCG, gallocatechin gallate. $^{*}p < 0.05$, $^{**}p < 0.01$.

more effectively than EGCG compared with that of Wy14643, PPAR-α agonist ($p < 0.01$) (Figure 3). Topical treatment with PPAR-α activators not only decreased cell proliferation in hyperproliferative epidermis but also increased the transformation of terminal differentiated keratinocytes into corneocytes, as witnessed by the elevated expression of involucrin, filaggrin and loricrine genes[19]. So, this result implies that GCG should be a more powerful agent for inducing PPAR-mediated-kerationocyte differentiation than EGCG and also implies that HE-EGCG-mix can be a new class of lead ingredient for recovery of skin damage because of its high content of GCG. Although, we assume that it might be caused by the different interactions of each epimer with the active site of PPAR-α, the structure-activity relationship studies are necessary for identifying why GCG is a potent and selective PPAR-α agonist more than EGCG[20,21].

4. Conclusion

Although the effects of topical application of catechin, rather than oral administration, may have a greater clinical significance in human beings, the animal studies suggest that oral administration of catechin can provide skin protection in rodents[6]. Therefore, it remains to be further clarified whether there is the same activity for skin protection by topical application in humanbeings, and also remains the mechanism of increased keratinocyte differentiation, recovery of the epidermal skin barrier and their association with multiple complex signaling mechanisms by topical administration in dermal skin. Also, for commercial uses, it is necessary that GCG shouldbe prepared to highly-purified-form by available method. Now, we are researching how to maximize and powderize GCG to highly-purified-form by convection-heating system using vaporizable solvent such as ethyl alcohol.

In conclusion, our study may provide a possibility that GCG could be made easily and cheaply by heat-epimerization method not by extraction method from green tea and could be a potentially effective agent for recovery of skin barrier by using catechin. We are pursuing further studies to determine how GCG affects these interlinked signaling pathways, how to make high content of GCG molecule easily and whether dietary GCG also improves skin barrier function or not.

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