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Evaluation of Skin Sebosuppression by Components of Total Green Tea (*Camellia sinensis*) Extracts

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Abstract In human beings, it is known that there is a correlation between the occurrence of acne and the ability to suppress sebum. Sebosuppression may be related to the inhibition of sebocyte proliferation, differentiation, and lipogenesis in sebaceous glands. To investigate the skin sebosuppressive activity of green tea extract, the *in vivo* effects of its flavonoid compounds on the androgen-dependent stimulation of pigmented macules in hamsters and performed *in vitro* experiments with human primary sebocytes were examined. Our results imply a dual activity of skin sebosuppression by green tea flavonoids; some catechins including epigallocatechin-3-gallate (EGCG) and gallocatechin-3-gallate (GCG) may reduce the differentiation of sebocytes by inhibiting PPAR-γ1 mRNA expression, whereas some flavonol glycosides including kaempferol may inhibit lipogenesis in sebaceous glands by decreasing levels of the mature form of sterol-sensitive response elements binding protein-1c (SREBP-1c). Therefore, green tea is a potentially effective material for use in the development of health foods or cosmetics for skin sebosuppression.

Key words: *Camellia sinensis*, catechin, flavonol glycoside, sebosuppression

Introduction

The tea plant *Camellia sinensis* has been cultivated in Asia for thousands of years. Currently, more than two-thirds of the world population consumes black tea or green tea (1). It has been shown that green tea contains various biologically active components with antioxidative and anticarcinogenic properties. Of these components, flavonoids including catechins and flavonols, which make up about 30% of the dry weight of green tea, are thought to be the most important. Green tea catechins are, structurally, primarily flavonols. The main catechins in green tea are (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epicatechin (EC). Green tea flavonols are a variety of glycosides of kaempferol, quercetin, and myricetin (2).

Reports of the ability of green tea and its associated catechins to act as antioxidants and radical scavengers, and to inhibit the growth cancer cells in culture and the action of androgens in experimental animals, raised the possibility that the consumption of green tea might lower the risk of cancer and androgen-dependent abnormalities (3). An ancient medicinal treatment also used green tea leaves as anti-inflammatory and anti-acne agents (3,4). In many organs, 5α-dihydrotesto-sterone (DHT) is converted from testosterone by 5α-reductase (5-AR) and can promote androgen-dependent diseases such as acne, prostate cancer, and baldness. Certain tea catechins, such as EGCG and ECG, are active 5-AR inhibitors (IC_{50} 10-20 μM) and are known to be beneficial for acne and seborrhea patients (4,5). In human beings, it is known that there is a correlation between the severity of acne and the ability to suppress sebum. Sebosuppresion therefore may be involved in the inhibition of sebocyte proliferation, differentiation, and lipogenesis in sebaceous glands (6). However, little attention has been given to tracing the action of green tea components and the mechanisms related to their sebosuppressive effect.

Skin sebaceous cells are specialized epithelial cells which terminally differentiate by accumulating neutral fat droplets until they burst, giving rise to the lipid-rich holocrine secretion, sebum (6,7). PPAR-α and PPAR-γ activators were found to stimulate lipid droplet accumulation in cultured immature sebocytes, but not in keratinocytes, as assessed histochemically using Oil Red O staining in cells cultured under high calcium conditions. PPAR-γ activation induced a greater degree of sebocyte colony differentiation than PPAR-α activation and significantly enhanced the differentiation of sebocytes in response to androgen. Thus, PPAR-γ plays a unique role in stimulating sebocyte differentiation (8).

Lipid homeostasis via sterol-sensitive response elements (SREs) in animal cells is achieved by a family of transcription factors called SRE-binding proteins (SREBPs) (9). SREBPs directly activate the expression of some 30-plus genes participating in the metabolism of mostly lipids, but also glucose. Activation of these originally membrane-bound transcription factors involves a proteolytic cascade through which the SREBP molecule is released from the membrane and obtains its mature form as a transcription factor (10). The active SREBP enters the nucleus and binds to those special DNA sequences, the SREs, in the promoter regions of many different genes. To date, 3 SREBPs have been identified; SREBP-1a and SREBP-1c are produced from a single gene through the use of alternate promoters, and SREBP-2 is transcribed from a separate gene. Most organs from adult animals, including liver, predominantly synthesize SREBP-1c and SREBP-2. Furthermore, SREBP-1c seems to be a mediator of dietary polyunsaturated fatty acid

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regulation and sebocyte lipogenesis although SREBP-1c is weaker than SREBP-1a and SREBP-2 due to its shorter transactivation domain (11).

The effects of green tea extracts on sebocyte differentiation and lipogenesis are not yet known. In this study, we examined several flavonoids from green tea for their influence on cell growth in hamster flank organs, inhibition of PPAR-γ1 expression, and decrease in the mature form of SERBP-1c protein in order to characterize the sebosuppressive effects of green tea extract and its flavonoid components during sebaceous gland maturation.

Materials and Methods

Reagents and sebocyte culture All reagents and solvents were purchased from commercial suppliers (Sigma-Aldrich-Fluka) and used without further purification. All other chemicals of analytical grade were purchased from Merck (Damstadt, Germany). Cell culture reagents were purchased from Invitrogen, Cambrex, Gibco, and Welgene. Primary human sebocyte cultures were obtained from a 53-year-old female who underwent an operation on the fronto-parietal facial area and were maintained without the use of a feeder layer in the culture medium [Dulbecco's modified Eagle medium (DMEM) and F12 media (3:1)] supplemented with 10^{-9} M cholera toxin (Calbiochem, Bad Soden, Germany) and 1.1×10^{-6} M hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained at 37°C in 5% CO_2 and were regularly subcultured at a split ratio of 1:3 using trypsin ethylenediamine tetraacetic acid 0.05/0.02%(w/v) in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}. Cells were harvested and analyzed 48 hr after each treatment by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analysis.

Measurement of green tea extract content Fresh tea leaves of *C. sinensis* were harvested in May 2005 on Jeju Island, Korea, and immediately processed into commercial steamed green tea as described by Hong *et al.* (12). Green tea extract was prepared by extraction for 24 hr in 95% ethyl alcohol at room temperature, and its flavonoid compounds were analyzed by the methods described by Choi *et al.* (13) and Ikegaya *et al.* (14). The samples were analyzed using a high performance liquid chromatography (HPLC) system (Waters 2695; Waters, Milford, MA, USA) connected to a ultra violet (UV)-VIS detector (Waters 2996 photodiode array detector) set at 280 nm. Identification and quantification of flavonols were achieved by comparing retention times and peak areas on the chromatograms with the references.

Growth inhibition assay of hamster flank organ macules Prepubescent male Syrian golden hamsters, castrated at 4 weeks-of-age, were obtained from Samtako Co. (Gyeonggi, Korea). Bilateral orchiectomy was performed under anesthesia. The hamsters were housed individually in plastic cages and maintained on a 12-hr light/12-hr dark cycle. Hamsters were divided into groups of 3-4 animals and used 1-2 weeks after castration. Hair on the lower back of each animal was shaved weekly with an electric hair clipper to expose the flank organs. A treatment solution (5 μL with ethanol as the vehicle) was applied topically to the flank organ once per day using a pipetteman and a polypropylene disposable tip. For each hamster, 2 flank organs were treated first with a solution containing testosterone and, 10 min later, with a solution containing the test materials. Growth of the flank organ was determined by measuring the length of the long axis of the pigmented spot (pigmented macule) with a digital-display caliper (Digimatic; Mitutoyo Co., Kanagawa, Japan). The surface area (mm^2) of the spot was calculated as the product of the long axis and the short axis (15). Experiments were repeated at least twice to assure reproducibility.

Semi-quantitative RT-PCR At the end of the experimental period, total cellular RNA was purified from cultured cells using TRIzol reagent (Gibco BRL, Grand Island, NY, USA). RNA was quantified by measuring the optical density at 260 nm and about 1 μg of total RNA was obtained from each sample. To synthesize oligo-dT_{15}-primed cDNA, total RNA was reverse transcribed into cDNA using a SuperScript™ III Reverse Transcriptase kit (Invitrogen, Groningen, Netherlands). The cDNA was subjected to PCR using an AccuPower PCR PreMix kit (Bioneer, Daejeon, Korea). The following PCR primers were synthesized by Bioneer: PPAR-γ1 sense oligonucleotide, 5'-TCTCTCCGTAATGGAAGACC-3', and anti-sense oligonucleotide, 5'-GCATTATGAGACATCCCCAC-3'; β-actin sense oligonucleotide, 5'-CAAGAGATGGCCACGGCTG CT-3', and anti-sense oligonucleotide, 5'-TCCTTCTGCAT CCTGTCGGCA-3'. The amplification products were predicted to be 474 and 300 bp for PPAR-γ1 and β-actin, respectively. PCR amplification was performed with a GeneAmp®PCR System 2700 (Applied Biosystems, Forster City, CA, USA) under the following conditions: denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 75 sec. The PCR products were electrophoresed on 1.5% agarose gels. Each DNA band was visualized by staining with ethidium bromide. As negative controls, we conducted PCR without RT and confirmed that there was no transcript amplification. To quantify the expression of the transcripts, the intensities of the PCR bands were measured by densitometry using Image-Pro Plus version 4.5 (10). The intensities were expressed relative to the β-actin intensities.

Western blot of mature SREBP-1 protein in sebocytes Protein lysate from sebocytes was prepared with cell lysis buffer (Cell Signaling Co., Denver, CO, USA) and the proteins were quantified by the BCA™ Protein Assay (Pierce, Rockford, IL, USA). Equal amounts of protein were electrophoresed on NuPAGE® Novex Bis-Tris-Gel (Invitrogen, Carlsbad, CA, USA) and transferred to a nitrocellulose membrane (LC2001; Invitrogen). SREBP-1c was detected with a goat polyclonal antibody (1:1,000 in 0.1% Tween 20 and nonfat milk) (MS-1207-P; Lab Vision, Fremont, CA, USA) and rabbit anti-goat IgG-HRP (Zymed, San Francisco, CA, USA). Actin was detected with a rabbit anti-actin antibody (A2066; Sigma-Aldrich). Blotting proteins were visualized by enhanced chemiluminescence (Amersham, Buchinghamshire, UK). Image analysis was performed using Image J version 1.34 (NIH, Bethesda, MD, USA).

Statistical analysis All data are expressed as the means

±SD. The statistical analyses were performed using the SPSS program (SPSS 12.0). One-way ANOVA and Duncan's multiple range test were used to examine the differences between groups. A p-values of 0.05 were considered to be statistically significant.

Results and Discussion

Analysis of flavonoid compounds in green tea extract Fresh green tea (*C. sinensis*) was harvested on Jeju Island in 2005, extracted with ethanol solvent, powderized by freeze-drying and the flavonoid components quantified using HPLC (Table 1). The contents of total catechins and flavonols in the powdered extract were approximately 293 and 25 mg/g powder, respectively. EGCG and kaempferol were the representative components for each group; both comprised no less than 40% of the total content of catechins and flavonols, respectively. Because of their high content, the powdered extract was appropriate to assess the efficacy of both EGCG and kaempferol.

Effect of flavonoid compounds on androgen-dependent stimulation of pigmented macules To investigate the skin sebosupressive activity of green tea extract, we examined the effects of its flavonoid compounds on the androgen-dependent stimulation of pigmented macules. Castrated hamsters were divided into groups of 4-6 animals. The flank organs were treated daily for 14 days with ethanol as the control or ethanol containing 0.5 μg testosterone (T) with or without 2 mg of GTE, EGCG, kaempferol, or flutamide as the reference. Kaempferol and GTE inhibited testosterone-induced growth of the pigmented macules by 72 and 56%, respectively, with kaempferol having as strong an effect as flutamide (Table 2). Inhibition of androgen-dependent flank organ growth by these compounds was evident from the fact that the pigmented macules on castrated animals treated with both an inhibitory compound and testosterone were lighter in color and smaller than those on animals treated with testosterone alone (Fig. 1a). The effect of flavonoids on the growth of sebaceous glands was examined histologically. The flank organs contained clusters of sebaceous glands (15). The lobules of the sebaceous glands in control skin were small and the sebocytes in the lobules stained with eosin. The flank organs from testosterone-treated skin contained distinctly large sebaceous lobules, but the effect of testosterone was reduced considerably by flavonoid treatment (Fig. 1b).

This finding suggests that flavonoids such as EGCG and kaempferol inhibit androgenic effects in hair follicles and sebaceous glands of the flank organ. The possibility that each flavonoid simply inhibited 5α-reductase (5-AR) is likely since flank organ growth is dependent on the local conversion of testosterone to DHT as in prostate growth in rodents and humans (4). However, this is not enough to explain why kaempferol had more potent growth inhibitory activity in the flank organ than EGCG, although EGCG was a more potent inhibitor (IC_{50} 10-20 μM) of 5-AR than kaempferol (IC_{50} >100 μM) (4). Therefore, it was necessary to characterize how each flavonoid should affect sebocyte maturation in terms of proliferation, differentiation, and lipogenesis.

Effect of flavonoid compounds on PPAR-γ1 mRNA expression in sebocytes A semi-quantitative RT-PCR assay was used to measure changes in PPAR-γ1 mRNA expression in human primary sebocytes. GTE and 2 catechins induced significant decreases (about 20 to 30% of the negative control) in PPAR-γ1 mRNA expression in sebocytes after 48 hr of treatment (Table 3), whereas kaempferol had no effect. This finding was in agreement with the fact that PPAR-γ activation induces a greater degree of sebocyte colony differentiation. Although catechins (EGCG and GCG) and flavonols (kaempferol and quercetin) have similar structures, kaempferol cannot inhibit PPAR-γ1 mRNA expression. This result implies that the gallate group in catechins may be important for inhibitory activity. Non-gallated catechins, such as EC or EGC, do not inhibit PPAR-γ1 mRNA expression (data not shown), suggesting that catechin structure, especially its trihydroxy phenol moiety, is important for the inhibition of PPAR-γ1 expression.

Table 1. Flavonoid composition of green tea extract (*Camellia sinensis*)

Flavonoid ingredients	Content (mg/g extract)[1)]	Quantity (%) in tea extract
Total catechins[2)]	293.4±4.6	29.3±0.4
EGCG	107.1±5.0	10.7±0.5
GCG	2.1±0.4	0.2±0.0
ECG	12.7±9.6	1.3±0.1
CG	7.7±3.7	0.8±0.3
EGC	131.7±6.7	13.2±0.7
Total flavonols	25.1±3.1	2.5±0.3
Kaempferol glycosides	12.8±2.8	1.3±0.3
Quercetin glycosides	8.3±1.1	0.8±0.1

[1)]Data are expressed in mg of flavonoids per g of powdered extract.
[2)]EGCG, epigallocatechin gallate; GCG, gallocatechin gallate; ECG, epicatechin gallate; CG, catechin gallate.

Table 2. Effects of green tea flavonoid on testosterone-stimulated growth of the pigmented macules of hamster flank organs

Treatment[1)]	Pigmented macule area (mm^2)	Inhibition (%)[2)]	p-Value[3)]
T (NC)	42.32±12.57	-	-
T+flutamide (PC)	16.32±7.22	61.4±17.1	<0.01
T+GTE	18.50±6.25	56.3±14.8	<0.01
T+EGCG	29.55±9.75	30.2±23.0	<0.05
T+GCG	19.85±8.61	53.1±20.3	<0.05
T+ kaempferol	11.63±7.21	72.5±17.0	<0.01

[1)]Flank organs were treated daily for 14 days with 5 μL ethanol containing 0.5 μg testosterone (T), with or without 1 mg of flutamide, green tea extract (GTE), epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), or kaempferol; NC, negative control; PC, positive control. Each group was comprised of 3 castrated male hamsters. At the end of the treatment period, the areas of the pigmented macules were determined and are presented as means±SEMs.
[2)]Percent decrease in the area of macules treated with catechins relative to the area of androgen-treated macules.
[3)]Significance of mean area of androgen-treated vs. catechin-treated macules by 1-way ANOVA with LSD test.

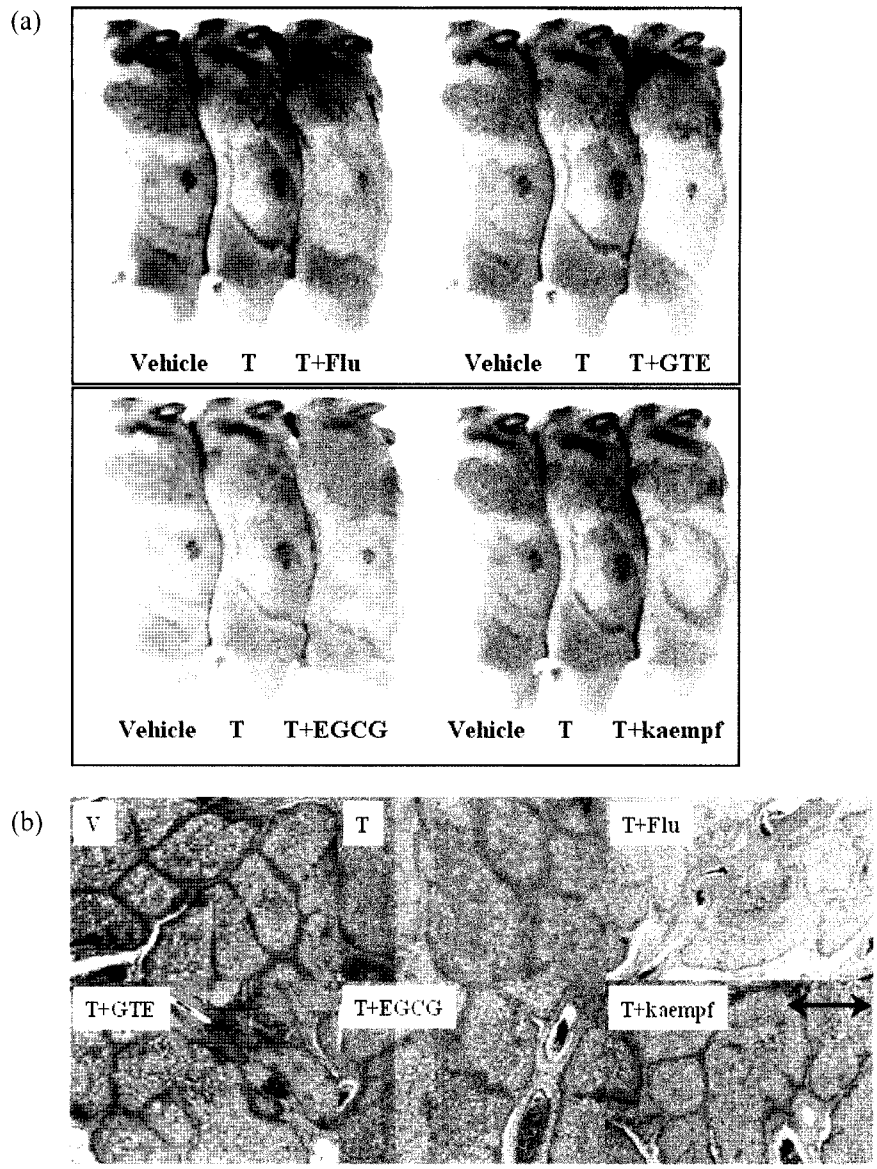

Fig. 1. Androgen stimulation and the effects of tea flavonoids. (a) Flank organs on both sides of castrated male hamsters were treated daily with ethanol alone (vehicle), 0.5 μg of testosterone alone (T), or 0.5 μg of testosterone with 2 mg of green tea extract (GTE), EGCG, or kaempferol (kaempf). Flutamide, an androgen receptor antagonist, was used as a reference. (b) Histological evaluation of hamster flank organs treated topically with testosterone alone or with flavonols. Flank organs were excised, fixed, sectioned, and then stained with hematoxylin and eosin (scale bar 300 μm).

Table 3. Effects of green tea ingredients on PPAR-γ1 mRNA expression in sebocytes

Treatment[1)]	Relative quantity (%) of PPAR-γ1[2)]
NC	100±2.6[b]
GTE	72.9±5.0[a]
EGCG	84.5±9.6[a]
GCG	78.2±3.7[a]
Kaempferol	99.0±2.2[b]

[1)]NC, negative control; GTE, green tea extract; EGCG, epigallocatechin gallate; GCG, gallocatechin gallate.
[2)]Data are expressed as the means±SD of triplicate experiments, and the significance of each mean was analyzed by 1-way ANOVA with Duncan's multiple test; values sharing the same superscript are not significantly different at $p<0.05$.

Effect of flavonoids on the maturation of SREBP-1c protein in sebocytes Western blot analysis was used to measure changes in the protein levels of SREBP-1c in human primary sebocytes. GTE, EGCG, and kaempferol reduced the levels of the mature form of SREBP-1c protein to 45, 74, and 61% of levels in testosterone-treated cells, respectively (Fig. 2). Among the 3 components, GTE exhibited the most potent inhibition of SREBP-1c maturation, and kaempferol inhibited mature SREBP-1c levels more so than EGCG. The SREBPs are members of a family of basic helix-loop-helix leucine zipper transcription factors that are known to be involved in regulating the expression of many enzymes involved in cholesterol and fatty acid metabolism (10,17). SREBP-1a controls the synthesis of fatty acids and cholesterol, while SREBP-1c is preferentially involved in the regulation of fatty acid

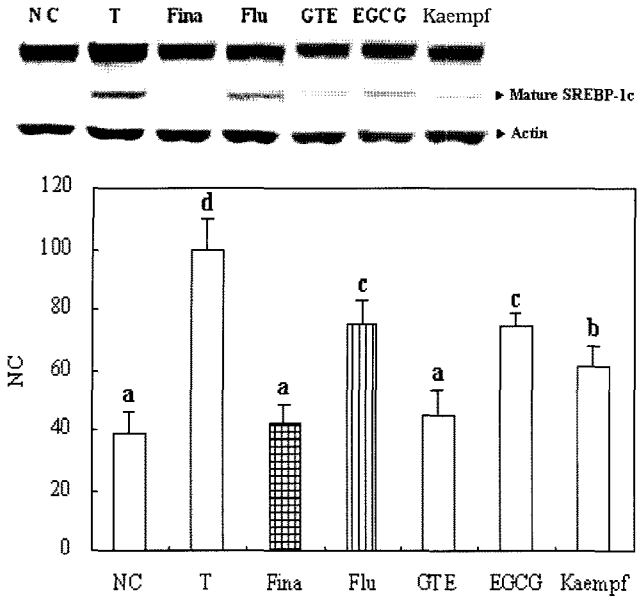

Fig. 2. Western blot assay of SREBP-1c protein in sebocytes. Finasteride, 5-α reductase inhibitor, and flutamide were used as references. Data are expressed as means±SDs of triplicate experiments. Values sharing a different superscript are significantly different at $p<0.05$.

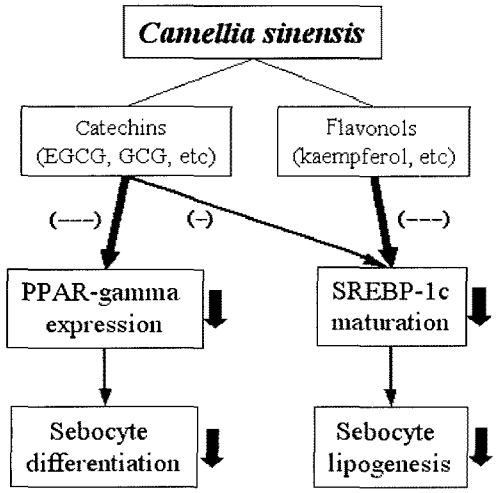

Fig. 3. Schematic representation of the dual activity of skin sebosuppression by *Camellia sinensis* ingredients. Of the various green tea components, some catechins including EGCG and GCG may reduce the differentiation of sebocytes by inhibiting PPAR-γ1 mRNA expression, whereas some flavonol glycosides including kaempferol may inhibit lipogenesis in sebaceous glands by decreasing the levels of the mature form of SREBP-1c protein.

synthesis. SREBP-2 is implicated in cholesterol metabolism. Rosignoli *et al.* (11) showed that (i) all SREBP isoforms are present in the sebaceous glands of hamster ear, (ii) androgen treatment increases the transcription of SREBP-1a and SREBP-1c but not SREBP-2, and (iii) DHT increases the levels of the mature form of SREBP-1. We suggested that the activation of SREBP-1c for sebum lipid synthesis is modulated by green tea extract. This hypothesis was confirmed by the inhibitory effect of GTE or kaempferol treatment on the maturation of SREBP-1c protein induced by testosterone.

Therefore, we suggest a dual activity in skin sebosuppression by *C. sinensis* ingredients as illustrated in Fig. 3. Of the various green tea components, some catechins including EGCG and GCG may reduce the differentiation of sebocytes by inhibiting PPAR-γ1 mRNA expression, whereas some flavonol glycosides including kaempferol may inhibit lipogenesis in sebaceous glands by decreasing the levels of the mature form of SREBP-1c.

Until now, the use of plant extracts to treat acne has been restricted to the anti-inflammatory or antibacterial effects of basil, pumpkin, and onion for topical application only (18). Onions possess antiallergic and anti-inflammatory effects due to the presence of flavonoids (quercetin and kaempferol) (19). Our results imply that total GTE should have a powerful sebosuppressive effect owing to the presence of both catechin and flavonol, suggesting that green tea could be a potentially effective material for development of new health foods or cosmetics for sebosuppression in skin.

Although the effects of the topical application of catechin rather than oral administration may have a greater clinical significance in human beings, animal studies suggest that the oral administration of catechin can provide skin protection in rodents (1,20). It remains to be seen if oral or topical administration in human beings results in the same sebosuppressive activity (21,22).

In conclusion, we have analyzed in detail the *in vivo* and *in vitro* effects of GTE and its flavonoids on the differentiation and lipogenesis of sebocytes. We also show, for the first time, that the dual sebosuppressive activity of green tea flavonoids is mediated by the inhibition of PPAR-γ1 transcription and inactivation of the SREBP pathway. However, additional studies are needed to determine whether the findings described in this report of a hamster model are applicable to human skin. The exact mechanism by which flavonoids modulate the inactivation of SREBPs in human sebaceous glands *in vivo* remains to be elucidated.

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