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Inhibition of sebum production and *Propionibacterium acnes* lipase activity by fullerenol, a novel polyhydroxylated fullerene: Potential as a therapeutic reagent for acne

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Synopsis

Oxidative stress plays a major role in acne formation; this suggests that oxygen-radical scavengers could be potential therapeutic agents. Fullerenol $C_{60}(OH)_{44}$, a recently developed polyhydroxylated fullerene, is a spherical carbon molecule that has many hydroxyl groups capable of potent radical-scavenging activity. We have investigated its inhibitory effects *in vitro* on sebum production in hamster sebocytes and in *Propionibacterium acnes* lipase activity. Sebum production was significantly reduced by 1.5 μM of fullerenol in cells that had been irradiated with 10 mJ/cm^2 UVB, although it was not altered in the non-irradiated cells, indicating that fullerene is a sebum suppressor for sebocytes under oxidative stress, such as that induced by UVB. It was also found that fullerenol has inhibitory activity against *P. acnes* lipase. These results suggest that fullerenol could be a beneficial skin care reagent for controlling acne vulgaris by suppressing sebum in the inflammatory response and by reducing *P. acnes* lipase activity.

INTRODUCTION

Acne vulgaris is a common inflammatory skin disease of the pilosebaceous follicles; it is characterized by a multifactorial pathogenesis that includes sebaceous gland hyperplasia, increased sebum production, hyperkeratosis of hair follicle pores, and *Propionibacterium acnes* colonization. However, previous studies have shown the major roles of oxidative stress in the pathophysiology of acne (1–6). Superoxide dismutase (SOD) activity is lower, and hydrogen peroxide generation increased (4) from the

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leukocytes of acne patients. Further, alteration of oxidative stress parameters such as catalase, glucose-6-phosphate dehydrogenase, SOD, and malondialdehyde (MDA) in the venous blood of acne patients has been reported, favoring a role of ROS in acne (5), and patients with severe acne reportedly show higher MDA levels in the skin tissues and plasma (6). From therapeutic aspects, tetracycline, erythromycin, and minocycline are preferable, compared with other antibiotics, because of their ability to suppress the generation of ROS from neutrophils (7,8). Moreover, although benzoyl peroxide (BPO), one of well-established acne treatments, exerts its anti-acne effect through antibacterial activity due to the production of ROS (9), it possess direct cytotoxic effects on leukocytes, conversely resulting in the inhibition of ROS generation by neutrophils in a dose-dependent manner (10). This evidence to support the major pathogenic roles of oxidative stress in acne suggests that oxygen radical scavengers, including fullerene and its derivatives, could be potential therapeutic agents.

Fullerene is a spherical carbon molecule that exhibits powerful radical-scavenging activity because of its unique "cage" structure. Consequently, the antioxidant activity of fullerene is several-hundred-fold higher than that of other antioxidants (11). Thus, fullerene has been reported to be beneficial in the treatment of neurodegenerative disorders (12) and arthritis (13–15); furthermore, it is expected to be applicable to various oxidative diseases (16). It has been reported that fullerene exhibits protective activity against keratinocyte apoptosis caused by the reactive oxygen species (ROS) that arise from UV exposure (17). Fullerene penetrates well into the epidermis (18) without skin irritation or toxicity (19,20), and its potential as a topical drug for acne vulgaris has been proposed (21). Indeed, our open clinical trial showed its efficacy on acne vulgaris (22). However, fullerene is problematic in biological applications because it has poor solubility in polar solvents such as H_2O. In recent studies, a polyhydroxylated fullerene derivative, fullerenol, which is produced by a simple synthesis method, has been shown to have excellent water-soluble properties (23,24). In addition, our previous studies have demonstrated that fullerenol exhibits antimicrobial activity against *P. acnes* in addition to its potent antioxidant activity (25–28). These discoveries have prompted us to investigate whether fullerenol could be useful for the treatment of acne vulgaris. In this study, we have examined the ability of fullerenol to suppress sebum production, and to inhibit the lipase activity of *P. acnes*.

EXPERIMENTAL

MATERIALS

Fullerenol was synthesized according to the method reported in a previous study (24). Hamster sebocytes, derived from the sebaceous glands of the auricles of five-week-old male golden hamsters, HuMedia-BB, and an assay kit for lipid production (SE-3001) were purchased from Kurabo Co. (Osaka, Japan). Clinically isolated *P. acnes* was obtained from Nicoderm Research Inc. (Osaka, Japan). GAM medium and adapalene were purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan) and Funakoshi Co. Ltd. (Tokyo, Japan), respectively. We purchased 4-methylumbelliferone (4-MU) and 4-MU-oleate (4-MUO) from Sigma (St. Louis, MO).

ASSAY FOR SEBUM PRODUCTION FROM HAMSTER SEBOCYTES

Hamster sebocytes were grown in HuMedia-BB supplemented with 8% fetal bovine serum (FBS), 2% human serum (HS), and 10 ng/ml hEGF. After reaching confluency, the culture was continued for a further seven days. The cells were then fed with HuMedia-BB supplemented with 8% FBS, 2% HS, and 10 μg/ml insulin to induce differentiation and thus sebum production, and simultaneously irradiated with UVB (10 mJ/cm^2). Every second day the medium was changed and fresh fullerenol solution was added. After incubation for 14 days, the cells were harvested and subjected to an assay for lipid production (SE-3001 kit) by using the "oil red" method according to the manufacturer's instructions; this allowed us to determine the sebum produced per cell. Sebum production from hamster sebocytes was statistically analyzed by the Student's *t*-test. Differences of $p < 0.05$ were considered significant.

PREPARATION OF *P. ACNES* LIPASE

Collected human sebum was suspended in boiled and de-aerated saline (10 ml). The suspension was placed on a GAM agar plate for the growth of anaerobic bacteria and cultured at 37°C for 48 h. A single colony of *P. acnes* was isolated and cultured in GAM liquid medium under the same conditions. The bacterial cells were collected by centrifugation (3,000*g*, 5 min, 4°C) and washed twice in 50 mM Tris-HCl (pH 7.4). The *P. acnes* cells were ruptured by ultrasonication and the obtained cell lysate was used as *P. acnes* lipase diluted to 100 μg/ml by Tris-HCl (50 mM, pH 7.4) (29).

MEASUREMENT OF LIPASE-INHIBITORY ACTIVITY

The inhibitory activity against *P. acnes* lipase was assessed by measuring the level of fluorescent 4-MU produced by lipase from the oleate ester, 4-MUO. The reaction mixture was 4-MUO (25 μl, 0.1 mM) and a sample solution (25 μl). *P. acnes* lipase (50 μl) was added to the reaction mixture (final volume, 100 μl). After the mixture was incubated at 37°C for 30 min, the amount of 4-MU released by the lipase was measured by using a fluorescence plate reader (SpectraMax Gemini, Molecular Devices Inc., Sunnyvale, CA) (excitation: 335 nm; emission: 460 nm). *P. acnes* lipase activity was determined as the amount of 4-MU released in 1 min (referred to a standard calibration curve for 4-MU) (29). The measurements were performed in triplicate, and half-maximal (50%) inhibitory concentration (IC_{50}) values were determined according to the following regression line formulae:

$$IC_{50}\ \text{value}(y) = b_1 x + b_0 \tag{1}$$

$$\text{Coefficient of regression } b_1 = \left[N\left(\sum x_i y_i\right) - \left(\sum x_i\right)\left(\sum y_i\right)\right] \Big/ \left[N\left(\sum x_i^{\,2}\right) - \left(\sum x_i\right)^2\right] \tag{2}$$

$$b_0 = \left[\left(\sum x_i^{\,2}\right)\left(\sum y_i\right) - \left(\sum x_i y_i\right)\left(\sum x_i\right)\right] \Big/ \left[N\left(\sum x_i^{\,2}\right) - \left(\sum x_i\right)^2\right] \tag{3}$$

RESULTS

IN VITRO SUPPRESSION OF SEBUM PRODUCTION IN HAMSTER SEBOCYTES

To examine whether fullerenol suppresses sebum production, we assayed sebum production from cultured hamster sebocytes because hamster sebocytes are useful model cells *in vitro,* with proliferation and lipid synthesis abilities similar to those of human sebocytes (30,31). As a result, we could not detect any significant effect on sebum production in cultured hamster sebocytes by 1.5 and 15 μM of fullerenol (Figure 1A). We then irradiated the cells with UVB as a model of oxidative stress in acne because previous studies showed that *in vivo* UVB irradiation causes sebaceous gland hyperplasia of hairless mice (32) and hamsters (33) and stimulates sebum production in cultured hamster sebocytes (34). Then, sebum production was stimulated by 10 mJ/cm^2 UVB irradiation (Figure 1B), consistent with a previous report (34), and so we studied the effect of fullerenol on sebum production in sebocytes stimulated by UVB irradiation. We found that 1.5 and 15 μmol/l of fullerenol suppressed sebum production (Figure 1C); fullerenol is thus a suppressor of sebocyte sebum expression, specifically under conditions of oxidative stress such as UVB irradiation.

LIPASE-INHIBITORY ACTIVITY

The inhibitory activities on *P. acnes* lipase by fullerenol and adapalene are shown in Figure 2. The IC_{50} values for adapalene and fullerenol were 197.6 μM and 837.1 μM, respectively. It was not possible to evaluate the effects of pristine fullerene by this method, as it acts as a quencher of 4-MU.

DISCUSSION

We have previously reported that, unlike pristine fullerene, fullerenol exhibits significant antimicrobial activity against several microorganism species, including *P. acnes.*

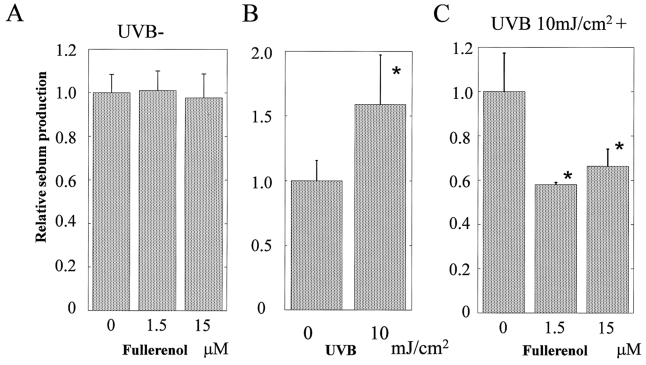

Figure 1. Effect of fullrenol on *in vitro* sebum production in hamster sebocytes. A. Hamster sebocytes were cultured in HuMedia-BB containing 8% fetal bovine serum, 2% human serum, and 10 μg/ml insulin (differentiation medium), with either 1.5 μM or 15 μM of fullerenol or control for 14 days. Sebum levels in cell lysate were determined as described in Experimental. B. Cells in the differentiation medium produced elevated levels of sebum when subjected to 10 mJ/cm^2 UVB. C. Cells were cultured in the differentiation medium while being subjected to 10 mJ/cm^2 UVB. Sebum production was significantly attenuated in cells whose medium was supplemented with fullerenol (1.5 μM and 15 μM, 14 days). $^*p < 0.05$.

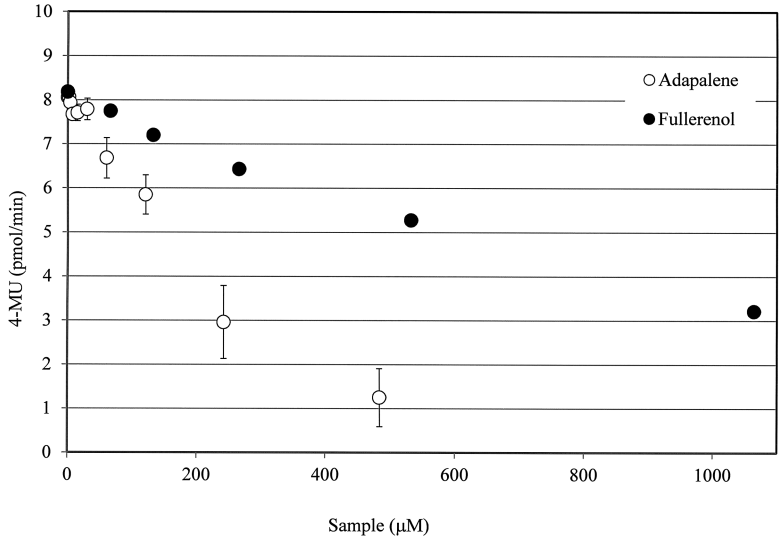

Figure 2. Dose-dependent inhibition of *P. acnes* lipase activity by fullerenol and adapalene. *P. acnes* lipase activity was determined by its conversion of 4-MUO into (fluorescent) 4-MU. The inhibitory activities of fullerenol and adapalene were measured at concentrations of 1.9–484.8 μM and 66.5–1050 μM, respectively.

Thus, it may be concluded that fullerenol is a more promising candidate for the treatment of acne vulgaris (25). Consequently, we investigated its inhibitory effect on sebocyte sebum production resulting from UV irradiation and on *P. acnes* lipase activity.

An *in vitro* assay of sebum production demonstrated that just 1.5 μM of fullerenol is sufficient to reduce sebocyte sebum production induced by UV irradiation. This result indicates that fullerenol is a sebum suppressor specifically under oxidative stress, such as UVB exposure. On the other hand, our previous study reported that 75 μM of polyvinylpyrrolidone (PVP)-wrapped pristine fullerene (PVP-fullerene) decreased sebum production by 27.4% by using the same method (22) but without UVB irradiation. Thus, fullerenol can be a sebum suppressor more specific for oxidative stress, compared with PVP-fullerene.

The IC_{50} value of fullerenol against *P. acnes* lipase activity was four times higher than that of adapalene. We previously reported that pristine fullerene in olive squalane was effective in the treatment of acne vulgaris in an open clinical study of 11 individuals (22). However, after application for eight weeks, inflammatory lesions were reduced by only 37.8%; this value is lower than that observed for adapalene gel in a previous study (median, 63.7%) (35).

It is known that antioxidant activity correlates well with a reduced severity of acne vulgaris (36). Fullerene and fullerenol are potent radical scavengers against ROS by ESR and the β-carotene breaching method (26–28). It is highly likely that fullerene and fullerenol might be effective antioxidants in clinical application. Therefore, we consider it prudent to test the effectiveness of fullerene in the reduction of inflammatory acne in an open clinical trial. Porphyrins, metabolic products of *P. acnes,* reportedly stimulate expression of keratinocyte-derived IL-8 in pilosebaceous tissue, resulting in perifollicular inflammation (37). Therefore, there is a need to evaluate the anti-inflammatory effects of fullerene

and fullerenol on acne vulgaris in order to improve our understanding of their molecular mechanism.

CONCLUSION

Our present data and existing findings suggest that fullerenol suppresses acne by inhibition of sebum production, by inhibition of *P. acnes* lipase activity, and by antimicrobial effect against *P. acnes* (possibly attributable to their antioxidant activity). We consider that both fullerene and fullerenol are suitable as cosmetic and/or "quasi-drug" active ingredients for acne sufferers following demonstration of their *in vivo* efficacy.

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