Involvement of *Propionibacterium acnes* in the Augmentation of Lipogenesis in Hamster Sebaceous Glands *In Vivo* and *In Vitro*

Katsuhiro Iinuma[1], Takashi Sato[1], Noriko Akimoto[1], Norihisa Noguchi[2], Masanori Sasatsu[2], Setsuko Nishijima[3], Ichiro Kurokawa[4] and Akira Ito[1]

Propionibacterium acnes is considered to be involved in the aggravation of acne vulgaris, but it remains unclear whether *P. acnes* directly influences lipogenesis in sebaceous glands. In this study, we showed that a culture medium of *P. acnes* (acnes-CM) and formalin-killed *P. acnes* (F-acnes) prepared from *P. acnes* strains, JCM6473 and JCM6425, intracellularly augmented lipid droplet formation and triacylglycerol (TG) synthesis in undifferentiated and insulin-differentiated hamster sebocytes. Acnes-CM and F-acnes prepared from four clinical *P. acnes* strains elicited the same lipogenesis augmentation. The augmented TG production resulted from an increase in the diacylglycerol acyltransferase activity. Topical application of acnes-CM to the skin of hamster auricles every day for 4 weeks revealed that sebum accumulation was augmented in sebaceous glands and ducts. Furthermore, both acnes-CM and F-acnes increased the production of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), a cytochrome P450 (CYP)-linked sebaceous lipogenic factor, in differentiated sebocytes. A CYP inhibitor, SKF-525A, decreased the acnes-CM- and F-acnes-augmented production of TG and 15d-PGJ_2. Thus, to our knowledge these results provide previously unreported evidence that *P. acnes* directly participates in the augmentation of sebaceous lipogenesis through a proposed mechanism in which an increase of 15d-PGJ_2 production through the CYP pathway is closely associated with the enhancement of TG production.

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INTRODUCTION

Propionibacterium acnes, a Gram-positive anaerobic microbial species, is considered to play a role in the aggravation of acne vulgaris, an inflammatory disease in sebaceous glands and pilosebaceous units in the skin (Bojar and Holland, 2004; Farrar and Ingham, 2004; Zouboulis *et al.*, 2005). The pathogenesis of acne is characterized by (i) excess sebum production and hyperplasia of sebaceous glands; (ii) formation of microcomedones, which is closely associated with hyperkeratinization of the follicular wall and infundibulum; and (iii) induction of inflammatory reactions, such as the acceleration of cytokine production and arachidonic acid metabolism in keratinocytes, sebocytes, and invaded inflammatory cells (Pawin *et al.*, 2004).

Regarding the involvement of endogenous factors in acne development, androgens, such as testosterone and 5α-dihydrotestosterone, have been reported to participate in excess sebum secretion and enlargement of sebaceous glands in acne lesions (Bialecka *et al.*, 2005). In addition, insulin, insulin-like growth factor 1, prostaglandins (PGs), and peroxisome proliferation-activating receptors have all been reported to contribute to the development and aggravation of acnes (Rosenfield *et al.*, 1999; Iwata *et al.*, 2005; Alestas *et al.*, 2006; Smith *et al.*, 2006; Trivedi *et al.*, 2006). Furthermore, it has been reported that the augmented production of proinflammatory cytokines, such as IL-1α, IL-6, IL-8, and tumor necrosis factor-α in epithelial cells and in infiltrated neutrophils and lymphocytes, is involved in the aggravation of acne and in the enhancement of inflammatory reaction in acne lesions (Vowels *et al.*, 1995; Chen *et al.*, 2002; Basal *et al.*, 2004; Graham *et al.*, 2004; Alestas *et al.*, 2006). These reports are intriguing in understanding the molecular mechanisms of acne pathology, as *P. acnes* is unlikely to be the direct causal bacteria responsible for acne in spite of the fact that it frequently exists within follicular canals (Farrar and Ingham, 2004). However, it remains unclear whether *P. acnes* could directly regulate sebum

[1]*Department of Biochemistry and Molecular Biology, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo, Japan;* [2]*Department of Microbiology, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo, Japan;* [3]*Nishijima Skin Clinic, Neyagawa, Osaka, Japan and* [4]*Department of Dermatology, Mie University Graduate School of Medicine, Tsu, Mie, Japan*

Correspondence: Dr Takashi Sato, Department of Biochemistry and Molecular Biology, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan. E-mail: satotak@ps.toyaku.ac.jp

Abbreviations: 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; acnes-CM, a culture medium of P. acnes*; CYP, cytochrome P450; DGAT, diacylglycerol acyltransferase; F-acnes, formalin-killed* P. acnes*; PBS(−), Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline; PG, prostaglandin; TG, triacylglycerol*

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production and inflammatory reactions in sebaceous gland cells (sebocytes).

In this study, we showed that a culture medium of *P. acnes* (acnes-CM) and formalin-killed *P. acnes* (F-acnes) derived from two types of *P. acnes* strains, JCM6425 and JCM6473, the augmented intracellular formation of lipid droplets in hamster sebocytes by increasing the *de novo* synthesis of triacylglycerols (TGs) *in vivo* and *in vitro*. In addition, similar results were obtained in treatment with acnes-CM and F-acnes prepared from clinical *P. acnes* strains. These results suggest a direct involvement of *P. acnes* in the augmentation of sebaceous lipogenesis through itself and/or through *P. acnes*-derived soluble factor(s).

RESULTS

The augmentation of lipid droplet formation and *de novo* TG synthesis by *P. acnes* in hamster sebocytes

We first examined whether *P. acnes* regulated sebaceous lipogenesis in cultured hamster sebocytes. As *P. acnes* has been isolated and characterized on human skin, but rarely on animal skin (Noble and Wade, 1998), we used acnes-CM and F-acnes, but not living *P. acnes*, in these experiments. As shown in Figure 1, both acnes-CM and F-acnes prepared from JCM6473 strain (1×10^6 cells) were found to augment the formation of intracellular lipid droplets in sebocytes (Figure 1b and c versus a). In addition, in insulin-differentiated sebocytes, in which the number of Oil Red O-positive cells was increased (Figure 1d versus a) (Akimoto *et al.*, 2005), both acnes-CM and F-acnes were found to enhance the accumulation of intracellular lipid droplets (Figure 1e and f versus d). Furthermore, both acnes-CM and F-acnes from the JCM6473 strain were found to increase the intracellular levels of TG, a major sebum component, in the undifferentiated (1.3- and 1.4-fold, respectively, at 1×10^6 cells) and differentiated sebocytes (1.2- and 1.3-fold, respectively, at 1×10^6 cells) (Table 1). The augmentation of TG levels resulted from an increase in the activity of diacylglycerol acyltransferase (DGAT), a rate-limiting enzyme for TG synthesis, by acnes-CM and F-acnes from JCM6473 strain (3.7- and 2.3-fold, respectively) in the differentiated cells (Figure 2). The increase of TG production and DGAT activity by acnes-CM and F-acnes was similarly detected in JCM6425 strain-treated undifferentiated and differentiated sebocytes (Table 1 and Figure 2). Moreover, as shown in Table 2, when undifferentiated hamster sebocytes were treated with acnes-CM and F-acnes prepared from four clinically isolated *P. acnes* strains, PA001–004, augmentation of TG production was observed. Thus, these results indicated that *P. acnes* directly increased sebaceous lipogenesis by augmenting DGAT-dependent *de novo* TG synthesis. As we confirmed that there was no difference in the regulation of lipid droplet formation and TG production by acnes-CM and F-acnes between strains JCM6473 and JCM6425, the former strain was used in the following experiments.

Augmentation of sebum accumulation in hamster sebaceous glands and ducts by *P. acnes in vivo*

We next examined whether *P. acnes* directly augmented sebum accumulation in sebaceous glands and ducts *in vivo*. When auricles of 3-week-old male golden hamsters were topically treated with acnes-CM at 1×10^6 cells in 95% ethanol/5% glycerol or with vehicle for 4 weeks and then stained with Oil Red O, the semiquantification analysis for Oil Red O-positive signals in the tissues revealed an increase of sebum accumulation in sebaceous glands and ducts (1.8-fold) (Figure 3). However, there was no change in

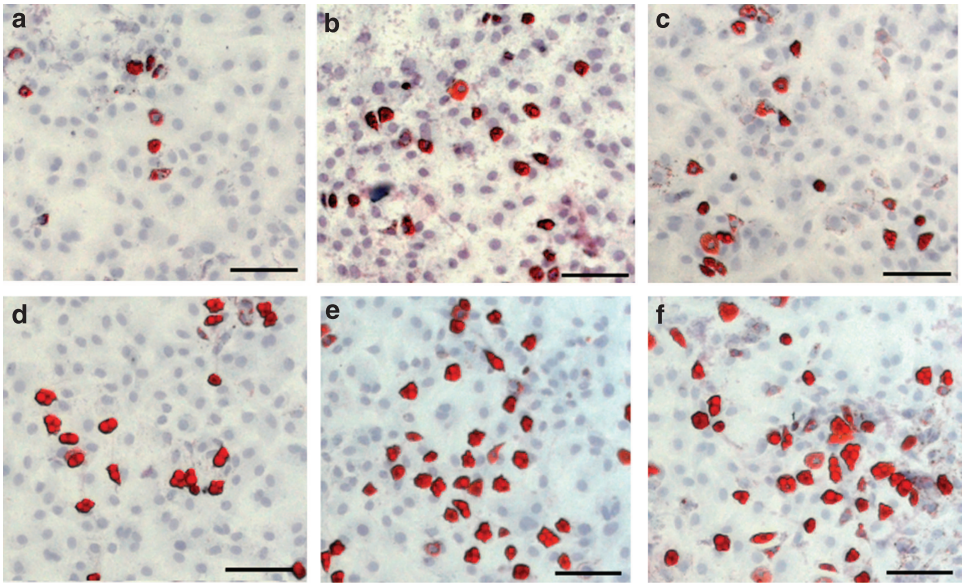

Figure 1. Augmentation of lipid droplet formation by *P. acnes* in hamster sebocytes. Hamster sebocytes at the third passage were treated every 3 days for 12 days without (**a** and **d**) or with acnes-CM (**b** and **e**) and F-acnes (**c** and **f**), which were prepared from JCM6473 strain at 1×10^6 cells in the presence (**d**–**f**) or absence of insulin (10 nM) (**a**–**c**). Cells were fixed and then stained with Oil Red O as described in the text. Bar = 50 μm.

Table 1. Augmentation of TG production by *P. acnes* in undifferentiated and differentiated hamster sebocytes

	TG levels (μg per μg DNA)			
	Undifferentiated sebocytes		Differentiated sebocytes	
Treatments (cell numbers)	Acnes-CM	F-acnes	Acnes-CM	F-acnes
JCM6473				
Control	1.70 ± 0.03	0.90 ± 0.09	3.65 ± 0.06	1.52 ± 0.02
1×10^5	2.01 ± 0.10***	1.15 ± 0.04*	4.04 ± 0.06**	1.91 ± 0.11**
1×10^6	2.23 ± 0.02***	1.24 ± 0.05**	4.37 ± 0.08***	2.00 ± 0.09**
1×10^7	1.78 ± 0.04*	1.10 ± 0.02*	3.85 ± 0.05*	1.92 ± 0.06***
JCM6425				
Control	1.72 ± 0.05	1.02 ± 0.08	3.60 ± 0.08	1.61 ± 0.01
1×10^5	1.98 ± 0.07**	1.10 ± 0.15	4.03 ± 0.09**	1.70 ± 0.09
1×10^6	2.19 ± 0.02***	1.21 ± 0.10*	4.24 ± 0.08***	1.83 ± 0.06**
1×10^7	1.79 ± 0.01*	1.17 ± 0.05*	3.87 ± 0.14*	1.72 ± 0.01***

Acnes-CM, culture medium of *P.* acnes; F-acnes, formalin-killed *P.* acnes; *P. acnes*, *Propionibacterium acnes*; TG, triacylglycerol.
Undifferentiated and differentiated hamster sebocytes at the third passage were treated every 3 days for 12 days with acnes-CM and F-acnes prepared from two *P. acnes* strains (JCM6473 and JCM6425) at the indicated cell numbers. After the treatments, intracellular levels of TG were measured as described in the text. Data are indicated as mean ± SD in triplicate dishes. Four independent experiments were reproducible and typical data are shown. *, **, and ***Significantly different from untreated cells (Control) ($P<0.05$, 0.01, and 0.001, respectively).

epidermal thickness in the acnes-CM-treated hamsters (Figure 3a and b), although hyperkeratinization in the follicular wall and infundibulum is characteristic of acne (Pawin *et al.*, 2004). Furthermore, we confirmed that the acnes-CM did not influence the cell proliferation in hamster sebocytes (data not shown). Therefore, *P. acnes* is likely to preferentially facilitate sebum production in hamster sebaceous glands *in vivo* and *in vitro*.

Involvement of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 in *P. acnes*-augmented TG production in the differentiated hamster sebocytes

Arachidonic acid metabolites have been reported to be associated with acne development and deterioration *in vivo* (Zouboulis, 2004; Alestas *et al.*, 2006). Recently, we reported that the augmentation of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) production is involved in the increase in TG production in hamster sebocytes (Iwata *et al.*, 2005). Thus, we examined whether *P. acnes* increased 15d-PGJ_2 production in hamster sebocytes. As shown in Figure 4, both acnes-CM and F-acnes were found to increase the production of 15d-PGJ_2 in these cells (1.8- and 1.6-fold at 1×10^6 cells, respectively). As we reported that 15d-PGJ_2 production in hamster sebocytes is dependent on the cytochrome P450 (CYP) pathway (Iwata *et al.*, 2005), we further examined whether the *P. acnes*-augmented TG and 15d-PGJ_2 production was inhibited by a CYP inhibitor, SKF-525A, in hamster sebocytes. As shown in Table 3, SKF-525A (20 μM) decreased the level of TG and the production of 15d-PGJ_2 in both acnes-CM- and F-acnes-treated cells. Therefore, these results indicated the close involvement of the CYP-15dPGJ_2 pathway in the *P. acnes*-augmented TG production in hamster sebocytes.

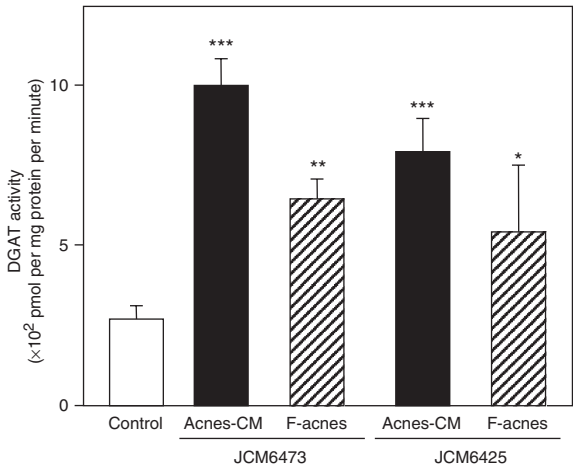

Figure 2. Increase in DGAT activity by *P. acnes* in the differentiated hamster sebocytes. The differentiated hamster sebocytes at the third passage were treated for 6 days with acnes-CM and F-acnes, which were prepared from two *P. acnes* strains (JCM6473 and JCM6425) at 1×10^6 cells. The harvested sebocytes were subjected to the measurement of DGAT activity as described in the text. Data are indicated as mean ± SD in three dishes. *, **, and ***Significantly different from untreated cells (Control) ($P<0.05$, 0.01, and 0.001, respectively).

DISCUSSION

The development of acne has been reported to be associated with the augmentation of not only androgen and proinflammatory cytokines (Vowels *et al.*, 1995; Farrar and Ingham, 2004; Pawin *et al.*, 2004; Zouboulis *et al.*, 2005) but also of PGs and peroxisome proliferation-activating receptor expression (Rosenfield *et al.*, 1999; Iwata *et al.*, 2005; Alestas *et al.*, 2006; Smith *et al.*, 2006; Trivedi *et al.*,

Table 2. Effect of clinical strains of *P. acnes* on the production of TG in undifferentiated hamster sebocytes

	Relative TG levels (fold versus untreated cells)	
Strains	**Acnes-CM (1×10^6 cells)**	**F-acnes (1×10^6 cells)**
PA001	1.99 ± 0.10***	2.12 ± 0.17***
PA002	1.33 ± 0.14**	2.45 ± 0.09***
PA003	1.99 ± 0.45**	2.45 ± 0.17***
PA004	2.02 ± 0.25**	1.01 ± 0.12

Acnes-CM, culture medium of *P.* acnes; F-acnes, formalin-killed *P.* acnes; *P. acnes, Propionibacterium acnes*; TG, triacylglycerol.
Undifferentiated hamster sebocytes at the third passage were treated every 3 days for 12 days with acnes-CM and F-acnes prepared from four clinical strains of *P. acnes* (PA001–004) (1×10^6 cells). After the treatments, intracellular levels of TG were measured as described in the text. The relative levels of TG were expressed by taking untreated cells as 1.00. Data are shown as means ± SD in three independent experiments using different hamster sebocytes. ** and ***Significantly different from untreated cells ($P<0.01$, and 0.001, respectively).

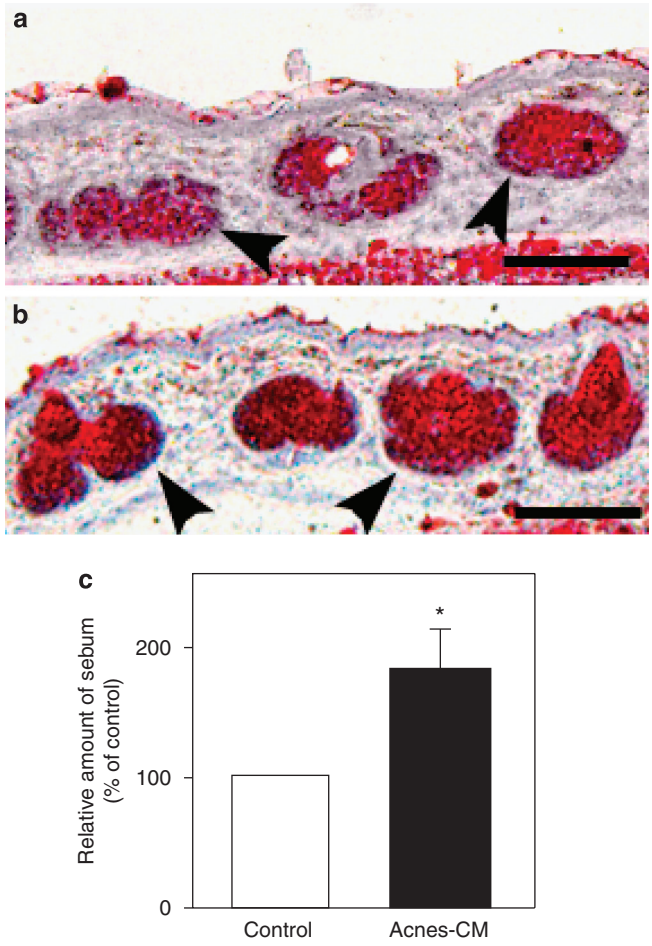

Figure 3. Effects of *P. acnes* on sebum accumulation in sebaceous glands in the skin of hamster auricles. Hamster auricles were treated every day for 4 weeks with acnes-CM (JCM6473) at 1×10^6 cells in 95% ehanol/5% glycerol or with vehicle solution (Control). The tissues were fixed and then stained with Oil Red O (**a** and **b**) as described in the text; panels a and b indicate vehicle- and acnes-CM-treated skin, respectively. Arrowheads and asterisks indicate sebaceous glands and epidermis, respectively. Bars = 300 μm. (**c**) The relative amounts of sebum accumulated in sebaceous glands were quantified from three individual areas of the skin treated with acnes-CM or vehicle (Control). Data are indicated as mean ± SD from three independent experiments. *Significantly different from the vehicle-treated hamsters (Control) ($P<0.05$).

2006), which subsequently contributes to the excess sebum secretion and production, and microcomedogenesis by hyperkeratinization of follicular wall and infundibulum (Pawin *et al.*, 2004). In this study, we showed that acnes-CM and F-acnes from two *P. acnes* strains (JCM6425 and JCM6473) directly augmented the formation of intracellular lipid droplets by increasing the DGAT-dependent TG synthesis in differentiated hamster sebocytes. In addition, we have confirmed that the gene expression of DGAT-1, but not DGAT-2 (Turkish and Sturley, 2007), is detectable in differentiated hamster sebocytes (data not shown). Furthermore, topical application of acnes-CM to the skin of hamster auricles was found to enhance sebum accumulation in sebaceous glands and ducts *in vivo*. At this time, this experimental density of *P. acnes* leading to the enhancement of lipogenesis could be supported by the finding that the skin surface density of *P. acnes* is in the range of 10^5–10^7/cm^2 on lipid-rich skin after puberty (Noble and Wade, 1998). Therefore, these results provide previously unreported evidence that *P. acnes* by itself exhibits a lipogenesis-stimulating activity in sebaceous glands. Moreover, this *in vivo* acne-like model may be useful for the pharmacological evaluation of anti-acne agents.

Neufang *et al.* (2001) have reported that the development of sebaceous glands with sebum accumulation is enhanced in transgenic mice overexpressing cyclooxygenase 2 in the epidermis. Recently, we reported that 15d-PGJ$_2$, the synthesis of which is dependent on the CYP pathway, is a sebocyte lipogenesis stimulator that augments the DGAT-mediated TG production (Iwata *et al.*, 2005). In this study, we have shown that both acnes-CM and F-acnes increased the production of 15d-PGJ$_2$, and a CYP inhibitor, SKF-525A, inhibited the augmented 15d-PGJ$_2$ production along with a decrease in intracellular TG levels in the differentiated sebocytes. Therefore, we propose the possibility that the CYP-dependent 15d-PGJ$_2$ production contributes to the augmentation of sebum production by *P. acnes* in sebaceous glands. Moreover, 15d-PGJ$_2$ has been reported to be an endogenous activator of peroxisome proliferation-activating receptor-γ (Kliewer *et al.*, 1995; Shibata *et al.*, 2002; Scher and Pillinger, 2005). A recent report by Trivedi *et al.* (2006) showed an increase in sebum secretion in patients treated with peroxisome proliferation-activating receptor ligands, such as thiazolidinediones and fibrates. Thus, these reports and our earlier study (Iwata *et al.*, 2005) support our hypothesis that *P. acnes*, at least partially, augments the production of 15d-PGJ$_2$, and thereby stimulates peroxisome proliferation-activating receptors to enhance TG production, which in turn causes excess sebum secretion as seen in acne lesions.

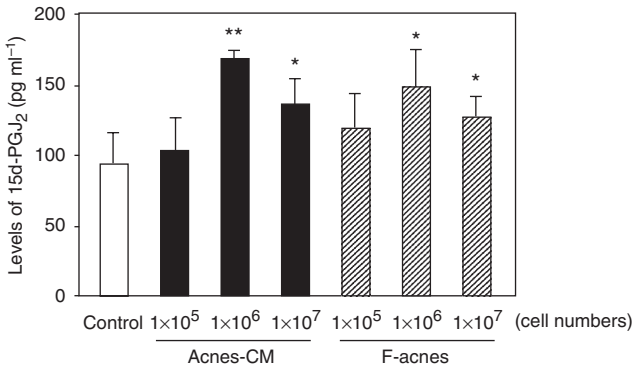

Figure 4. Augmentation of 15d-PGJ$_2$ production by *P. acnes* in differentiated hamster sebocytes. Differentiated hamster sebocytes at the third passage were treated every 3 days for a total of 12 days with acnes-CM and F-acnes, which were prepared from JCM6473 strain at the indicated cell numbers. After the treatments, 15d-PGJ$_2$ levels in the harvested culture media were measured as described in the text. Data are indicated as mean ± SD in triplicate dishes. * and **Significantly different from untreated cells (Control) ($P<0.05$ and 0.01, respectively).

Table 3. Inhibition of *P. acnes*-augmented 15d-PGJ$_2$ and TG production by a CYP inhibitor, SKF-525A, in differentiated hamster sebocytes

Treatments	Levels of 15d-PGJ$_2$ (pg ml^{-1}) (fold versus control)	Levels of intracellular TG (μg per μg DNA) (fold versus control)
Control	139.83 ± 21.28 (1.00)	2.58 ± 0.35 (1.00)
Acnes-CM (1×10^6 cells)	311.60 ± 5.34** (2.23)	3.61 ± 0.09** (1.40)
Acnes-CM (1×10^6 cells)+ SKF-525A (20 μM)	125.45 ± 19.20$^{\#\#}$ (0.90)	2.62 ± 0.44$^{\#\#}$ (1.02)
F-acnes (1×10^6 cells)	389.21 ± 49.77** (2.78)	3.60 ± 0.33** (1.40)
F-acnes (1×10^6 cells)+ SKF-525A (20 μM)	190.38 ± 4.89$^{\#}$ (1.36)	3.04 ± 0.19$^{\#}$ (1.18)
SKF-525A (20 μM)	142.37 ± 19.01 (1.02)	2.72 ± 0.49 (1.05)

Acnes-CM, culture medium of *P.* acnes; 15d-PGJ$_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$; F-acnes, formalin-killed *P.* acnes; *P. acnes*, *Propionibacterium acnes*; TG, triacylglycerol.
Differentiated hamster sebocytes at the third passage were treated every 3 days for 12 days with acnes-CM and F-acnes, which were prepared from JCM6473 strain at 1×10^6 cells in the presence or absence of a CYP inhibitor, SKF-525A (20 μM). After the treatments, the intracellular TG levels were measured as described in the text. Data are indicated as mean ± SD in triplicate dishes. **Significantly different from untreated cells (Control) ($P<0.05$). $^{\#}$ and $^{\#\#}$Significantly different from acnes-CM- or F-acnes-treated cells ($P<0.05$ and 0.01, respectively).

P. acnes-mediated sebaceous inflammation and hyperkeratinization in the follicular wall have been considered to be caused by the bacterial lipase-degraded lipid products (Pawin *et al.*, 2004), and by peptidoglycan polysaccharides, a component of the Gram-positive bacteria cell walls (Vowels *et al.*, 1995; Graham *et al.*, 2004), through Toll-like receptor 2 (Kim, 2005; McInturff *et al.*, 2005; Nagy *et al.*, 2005). Presently, we have not identified soluble and/or cell-associated factor(s) derived from *P. acnes* that may facilitate sebum synthesis in sebaceous glands. Taken together with a recent report of Oeff *et al.* (2006) that human SZ95 sebocytes constitutively express Toll-like receptor 2, our finding that F-acnes increased TG production in hamster sebocytes allows us to speculate that peptidoglycan polysaccharides of *P. acnes* may be associated with the augmented lipogenesis. Further experiments are needed to clarify the mechanism of *P. acnes*-enhanced sebaceous lipogenesis, for example, the involvement of peptidoglycan polysaccharides of *P. acnes*.

In general, *P. acnes* is confined to the infundibula of pilosebaceous units in healthy skin (Bojar and Holland, 2004; Pawin *et al.*, 2004). The onset of acne has been associated with an increase of *P. acnes* proliferation, which correlates with the augmented supply of TG, a source nutrition of *P. acnes*, from sebaceous glands (Bojar and Holland, 2004; Pawin *et al.*, 2004). Taken together with our findings, although it is controversial whether *P. acnes* by itself interacts with sebaceous glands, we propose that an aggravation mechanism of acne exists whereby soluble factor(s) released from hyperproliferated *P. acnes* diffuses through sebum in the sebaceous duct and reaches the sebaceous glands and pilosebaceous units from a distance, whereby sebaceous lipogenesis and inflammatory reactions mediated by infiltrated immune cells are enhanced in acne lesions.

In conclusion, we have shown that *P. acnes* augmented sebum production and accumulation by increasing DGAT-mediated TG synthesis in sebaceous glands *in vivo* and *in vitro*. In addition, the increase in 15d-PGJ$_2$ production by *P. acnes* was associated with the augmentation of lipogenesis in hamster sebocytes. Therefore, these results provide previously unreported evidence in the pathology of acne that *P. acnes* directly participates in the augmentation of sebaceous lipogenesis by *P. acnes*-derived soluble factor(s) and/or *P. acnes* itself, by the mechanism that the increase in 15d-PGJ$_2$ production through CYP pathway(s) may be closely associated with the enhancement of TG production.

MATERIALS AND METHODS

P. acnes culture conditions

Two stains of *P. acnes* (JCM6425 and JCM6473) were purchased from RIKEN BioResource Center (Ibaraki, Japan). In addition, four clinical *P. acnes* strains (PA001–004) were prepared from individual acne patients according to the method of Kishishita *et al.* (1979). *P. acnes* was cultured in GAM medium (Nissui Pharmaceutical, Tokyo, Japan) or DMEM/Ham's F12 medium(DMEM/F12) (1:1; Invitrogen, Carlsbad, CA) for 72 hours at 37 °C under anaerobic conditions, and then centrifuged for 15 minutes at 5,000 *g* at 4 °C. The precipitated bacteria were washed three times with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline [PBS(−)] and then treated with 4% formaldehyde/PBS(−) for 30 minutes. After an additional centrifugation, F-acnes was re-suspended in distilled water, and then aliquoted and stored at −20 °C. Acnes-CM was filtered, and then similarly aliquoted and stored at −20 °C. The number of *P. acnes* was determined by plating serial dilutions of the culture onto a GAM agar plate.

Cell culture and treatments

Hamster sebocytes were established from sebaceous glands of auricles of 5-week-old male golden hamsters, as described earlier (Sato *et al.*, 2001). Sebocytes (1.8×10^5 cells/dish) in DMEM/F12 supplemented with 6% heat-denatured fetal bovine serum (JRH Bioscience, Tokyo, Japan), 2% human serum (ICN Biochemicals, Costa Mesa, CA), 0.68 mM L-glutamine (Invitrogen), and recombinant human epidermal growth factor (10 nM) (Progen Biotechnik GmbH, Heidelberg, Germany) were plated onto 35-mm-diameter culture dishes (Becton Dickinson, Tokyo, Japan) for 24 hours to achieve complete cell adhesion. The cells (undifferentiated sebocytes) were treated every 3 days for up to 12 days with acnes-CM, F-acnes (corresponding to 1×10^5–10^7 cells), or vehicle in DMEM/F12 supplemented with heat-denatured fetal bovine serum, human serum, and L-glutamine. In addition, when sebocytes were pretreated with insulin (10 nM) (Sigma Chemical, St Louis, MO) for 7 days, the cells were differentiated to form intracellular lipid droplets, as described earlier (Iwata *et al.*, 2005). The differentiated sebocytes were similarly treated with acnes-CM, F-acnes, or vehicle in the same medium in the presence or absence of a CYP inhibitor, SKF-525A (20 μM) (Sigma Chemical), for 12 days. In this series of experiments, hamster sebocytes were used as far as the third passage level.

Histochemical analyses

Auricles of 3-week-old male golden hamsters were topically treated every day for 4 weeks with acnes-CM (corresponding to 1×10^6 cells) in 95% ethanol/5% glycerol, or with the same volume of vehicle. After treatment, the tissues were fixed with 4% paraformaldehyde/PBS(−), embedded in paraffin, and then their sections were subjected to Mayer's hematoxylin and eosin staining (Wako Pure Chemicals, Osaka, Japan). After completing the staining, tissue sections were washed with ethanol and xylene, and then viewed with a light microscope furnished with a digital camera (Olympus, Tokyo, Japan). Epidermal thickness in six randomly chosen areas per section was measured under a light microscope at 100-fold magnification, as described earlier (Tanaka *et al.*, 2004). The animals had free access to food and water according to the Guidelines of Experimental Animal Care issued by the Prime Minister's Office of Japan. The experimental protocol was approved by the Committee of Animal Care and Use of Tokyo University of Pharmacy and Life Sciences.

Oil Red O staining

Skin samples from auricles of hamsters treated with acnes-CM or vehicle were snap-frozen in liquid nitrogen, and the frozen tissues were then sectioned into 8-μm-thick sections, which were in turn washed in distilled H_2O and incubated in 60% isopropanol. The tissue sections were stained with 0.3% Oil Red O (Sigma Chemical) in isopropanol/distilled H_2O (3:2, vol:vol) at 37 °C for 15 minutes, and then viewed with a light microscope furnished with a digital camera (Akimoto *et al.*, 2005). The sections were also counterstained with Mayer's hematoxylin solution (Wako Pure Chemicals). Relative amounts of the Oil Red O-stained sebum in sebaceous glands and ducts were quantified from three individual areas per tissue using an image analysis system, Lumina Vision (Ver. 2.2.2; Mitani, Fukui, Japan) (Kuwahara *et al.*, 1999; Fan *et al.*, 2001). Cultured sebocytes were similarly stained with Oil Red O and Mayer's hematoxylin solution.

Measurement of intracellular TG

The harvested sebocytes were sonicated at 4 °C, and then levels of intracellular TG in the cell lysate were measured using Liquitech TG-II (Roche Diagnostics, Tokyo, Japan) at 590 nm, as described earlier (Akimoto *et al.*, 2005). The amount of TG was calculated using an authentic trioleinate-standard solution (0.6 mg ml^{-1}). In addition, the intracellular DNA content was measured using authentic salmon sperm DNA (6.25–100 μg ml^{-1}) and 3,5-diaminobenzoic acid dihydrochloride (Sigma Chemical), as described earlier (Sato *et al.*, 2001).

Measurement of DGAT activity

DGAT activity in hamster sebocytes treated with acnes-CM and F-acnes was measured using 1,2-dioleoyl glycerol (Cayman Chemical, Ann Arbor, MI) and [^{14}C]palmitoyl-CoA (Amersham Biosciences, Tokyo, Japan), as described earlier (Iwata *et al.*, 2005).

Enzyme immunoassay for 15d-PGJ$_2$

The levels of 15d-PGJ$_2$ in the harvested culture medium were measured using enzyme immunoassay kits for 15d-PGJ$_2$ (Assay Designs, Ann Arbor, MI) according to the manufacturer's instructions.

Statistical analysis

Data are presented as means ± SD, and were analyzed by a one-way analysis of variance and by the Fisher test for multiple comparisons. A value of $P<0.05$ was considered to indicate a statistically significant difference.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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