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천연물 유래의 5α-Reductase 저해제의 개발과 인체 유래 피지선 세포의 배양을 이용한 피지분비 억제 효과 측정

정 세 규† · 김 정 기 · 백 지 훈 · 이 기 무 · 조 인 식 · 이 승 훈*

(주)애경 중앙연구소, *연세대학교 의과대학 피부과
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5α-Reductase Inhibitors from Native Plants and their Sebosuppressive Effects in Cultured Human Sebaceous Gland Cells

Se Kyoo Jeong†, Jeong Kee Kim, Jihwoon Baek, Ki Moo Lee, In Shik Cho, and Seung Hun Lee*

*Central Research Laboratories, Aekyung Co. Ltd., 217-2 Sinseung-dong Yuseong-gu, Daejeon 305-345, Korea
**Department of Dermatology, Yonsei University College of Medicine
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요 약: 본 연구에서는 피지분비 억제 효과를 지닌 신규 화장품 원료를 대한민국 유래의 천연 식물에서 개발하고자 하였다. 다양한 식물 추출물의 5alpha-reductase에 대한 저해효과를 자외선 분광광도계를 이용한 효소 동력학적 분석법을 통하여 평가하였다. 효소 동력학적 분석을 위하여 각각 쥐의 간 조직과 배양된 인체 유래 피지선 세포에서 5alpha-reductase를 추출하였다. 그 결과, 5-AR에 대하여 뛰어난 저해효능을 보이는 3종의 식물 추출물을 선정하였고, 이들의 피지분비 억제 효과를 배양된 인체 유래 피지선 세포를 이용하여 분석하였다. 연구의 결과, 수십 종의 식물 추출물 중 해송 추출물이 가장 강력한 5-AR 저해능을 나타내었으며, 인체 유래 피지선 세포에 대하여 0.005% 농도로 처리한 경우 48%의 피지 생성량 감소 효과를 나타내었다. 해송 추출물은 피지 분비를 감소시켜 여드름이나 지루성 피부염과 같은 피부 질환에 효과를 지닌 화장품의 원료로 사용될 수 있을 것이다.

Abstract: This study was performed to develop new cosmeceutical agents with sebosuppressive activity from native plant extracts in Korea. Inhibitory effects of the extracts on 5α-reductase (5-AR) were evaluated by enzyme kinetics analysis using UV-spectrophotometric method. Two kinds of enzyme suspensions as 5-AR sources were prepared from rat liver tissue and cultured hSG cells. The sebosuppressive effects were determined by measuring the total lipid quantity produced in cultured hSG cells after incubation with the extracts. As a result, *Pinus thunbergii* extracts showed the most potent 5-AR inhibitory effects. Its K_i values were 0.0002% and 0.0014% for rat liver 5-AR and human sebaceous gland 5-AR, respectively. Addition of *Pinus thunbergii* extract to hSG cells showed 48% reduction in total lipid production at 0.005% concentration. In conclusion, *Pinus thunbergii* extracts can be used as a cosmeceutical agent to regulate sebum production and to alleviate the sebum-involved skin diseases, such as acne and seborrheic dermatitis.

Keywords: *Pinus thunbergii extract, 5α-reductase, sebaceous gland derived cells, sebosuppressive activity*

1. Introduction

The sebaceous gland, usually associated with a hair follicle, is a characteristic feature of the mammalian skin. While the exact function of the sebaceous gland and sebum in human is not yet fully understood, it is assumed that its role is to lubricate and waterproof the formed hair. It is also suggested that the sebaceous gland is very important for skin homeostasis, since the sebum is involved in the molecular organization of stratum corneum intercellular lipids [1] and transportation of antioxidants to skin surface [2]. In addition, sebaceous gland provides platelet-activating factor acetylhydrolase II, which protect the skin against oxidative stress [3]

† 주 저자 (e-mail: algeny@aekyung.co.kr)

and sebum-specific lipids (C16 : 1△6) shows an innate anti-microbial activity [4]. Recently, Fluhr *et al* also reported that sebaceous gland-derived glycerol is a major contributor to stratum corneum hydration [5]. Although a variety of functions have been proposed for cutaneous sebaceous gland-derived lipids, the most predominant view suggests that the principal role of sebum in human is negative, i.e. its well-accepted pathophysiological role in the provocation of acne. As a result, significant attempts have been tried to develop a new cosmeceutical agent, that has a regulatory effect on sebum production.

Through the decades of investigation, it has been resolutely established that the development and secretory activity of sebaceous glands are strongly influenced by hormones, especially androgens [6,7]. At the same time, it is also demonstrated that the sebaceous glands dominantly regulate the cutaneous androgen production [8]. Androgens act on the sebaceous gland by stimulating mitosis and lipogenesis, and the most effective forms are those with a 17β-hydroxy group, such as testosterone, 5α-dihydrotestosterone (DHT). Using the hypophysectomised, castrated rats, it has been reported that DHT has more potent stimulating activity on sebum production than testosterone [9]. Production of DHT from testosterone in sebaceous gland is considered as being catalyzed by 5α-reductase (5-AR). In human, two isoforms of 5-AR is presented. The type 1 isozyme has an optimal pH of 6~9 and represents the cutaneous type, which is mainly located in sebocytes and epidermal and follicular keratinocytes. The type 2 isozyme has an optimal pH of 5.5 and located mainly in the epididymis, seminal vesicles, prostate as well as in the inner root sheath of the hair follicle [10]. Since the activity of 5-AR is significantly elevated in hyperplastic prostates, male bold scalps and acne-bearing skin, as well as in acne-prone skin, inhibition of the 5-AR system appears to be a promising target for treatment of androgen-dependent skin disorders. Extensive researches have been done to develop an agent with 5-AR inhibitory activity and finasteride, which has a higher affinity for the type 2 isozyme, is the first clinically introduced one. Since the skin mainly express type I isozyme, however, the development of a new agent which specifically inhibit the type 1 isozyme may be of great importance for anti-androgenic treatment of seborrhea and acne.

In this study, we tried to develop a new agent with 5-AR inhibitory effects from various natural plants in Korea. Organic solvent extracts from natural plants were tested for their inhibitory effects, as well as sebosuppressive effects on human sebaceous gland derived cells.

2. Materials and Methods

2.1. Materials

Various plant extracts were obtained from Plant Extract Bank of Plant Diversity Research Center (Daejeon, Korea). Finasteride, testosterone, NADPH2, azelaic acid, nordihydroguaiaretic acid (NDGA) and genestein were purchased from Sigma chemicals (St. Louis, MO, USA). All the other reagents, unless otherwise mentioned, were of analytical grade.

2.2. Preparation of 5-AR Suspensions

Mature Sprague-Dawley female rat (7~8 weeks olds), purchased from Samtako (Kyunggi-do, Korea), were sacrificed by cervical dislocation. The liver was dissected and freed from fat, and homogenized in buffer solution (0.13 M sucrose, 10 mM Tris-HCl, 40 μL/L 2-mercaptoethanol) by homogenizer. The homogenate was centrifuged at 105000 G for 1 h (Optima, Beckman Coulter, Fullerton, CA, USA). The supernatant fraction was collected and used as a rat liver 5-AR suspension.

In order to prepare type 1 isozyme-dominant 5-AR suspension, human sebaceous gland derived cells were used. Isolation and *in vitro* culture of the sebaceous gland cells were done as previously described [11]. Briefly, sebaceous gland attached epidermis was separated from dermis using dispase (Invitrogen, Carlsbad, CA, USA) and the glands were carefully excised with 27 G needles under microscopic observation. The isolated glands were placed in culture dish and complete keratinocyte growth medium (Cambrex, East Rutherford, NJ, USA) was added. When the cells formed colonies around glands, they were subcultured using trypsin-EDTA solution. After 2~3 passages, the cells were harvested and cellular homogenate was prepared with buffer (10 mM potassium phosphate, 150 mM KCl, 1 mM EDTA) using homogenizer. The homogenate was centrifuged at 20000 G for 15 min and the supernatant was used as a sebaceous gland cell originated 5-AR

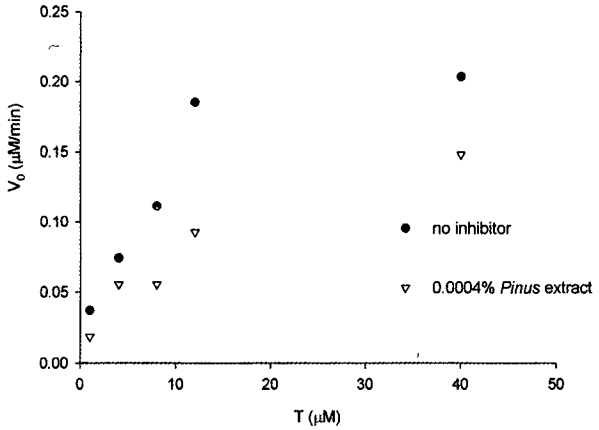

Figure 1. 5-AR reaction rate inhibited by *Pinus* extracts. T is the concentration of testosterone and V_0 is the reaction rate of 5-AR.

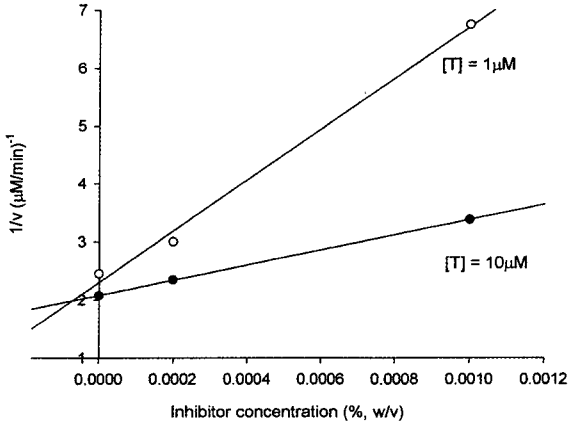

Figure 2. Dixon plot of *Pinus* extract. The x-axis value of the intersection point between two lines is calculated as K_i value. The reaction rate for two different concentration of testosterone (○ : [testosterone] = 1 μM, ● : [testosterone] = 10 μM) was measured at each concentration of inhibitors.

suspension.

2.3. Measurement of 5-AR Activity

Reaction mixture contains 22 μM $NADPH_2$ and 20 μL of enzyme suspension in 100 mM Tris-HCl buffer (pH 7.0). 20 μL of test substance was added to the reaction mixture and the reaction was initiated with the addition of the enzyme suspension. The reaction rate was calculated by measuring the concentration of $NADPH_2$ in the mixture, which was measured using UV-spectrophotometer (λ_{max}=340 nm)[12].

2.4. Measurement of Sebosuppresive Effects.

Human sebaceous gland derived cells were seeded to 24-well culture plate and maintained in complete keratinocyte growth medium. The sebosuppressive effect of selected plant extracts was evaluated by measuring the total lipid quantity after co-incubation with the extract for 24 h. After incubation, cells were washed twice with Ca^{++}, Mg^{++}-free PBS solution and harvested with trypsin-EDTA solution. The chloroform : methnol (2:1) was added to the cells and sonicated three times for 1 min. The solution was filtered through filter and the filtrates were evaporated to dryness under nitrogen stream. The weight of the total lipid was measured by analytical balance and the lipid residues were redissolved in chloroform and stored at -70°C for further analysis [10].

3. Results and Discussion

3.1. Enzyme Kinetics Analysis

Based on the $NADPH_2$ consumption rate measurement, 5-AR reaction kinetics analysis was performed to evaluate the inhibitory effects of natural extracts. Typical reaction plot is shown in Figure 1. The inhibitory effects were expressed as inhibition constant (K_i), which means the concentration of the substance at which 50% of the maximum reaction velocity (V_{max}) is shown. In order to calculate the K_i values, Dixon plots for each tested natural extracts were plotted. Typical Dixon plot is shown in Figure 2.

3.2. Inhibition of 5-AR by Natural Plant Extracts

The K_i values for tested natural plant extracts and finasteride and azelaic acid, both served as positive controls, are shown in Table 1. Among the tested substances, 3 kinds of plant extracts with highest inhibition activity were chosen and further investigated using 5-AR from human sebaceous gland derived cells. The K_i values for selected substances for 5-AR from human sebaceous gland derived cells are shown in Table 2.

3.3. Sebosuppressive Effects of Natural Plant Extracts

The sebosuppressive effects were evaluated by mea-

Table 1. K_i Values of Various Natural Plants Extracts in Korea for Rat Liver 5-AR

Materials	K_i
Pinus thunbergii (leaf)	0.0002%
Glycyrrhizae radix (root)	0.0002%
Actinostemma lobatum (whole)	0.0004%
Sophorae radix (root)	0.0009%
Artium lappa (leaf, stalk)	0.0011%
Trichosanthes kirilowii (var. japonica)	0.0021%
Ulmus parvifolia (stele)	0.0029%
Armeniaceae semen (seed)	0.0048%
Oenothera odorata (leaf)	0.0087%
Rhei radix et Rhizoma (root)	0.005%
Hoelen	0.036%
Portulacae Herba	N.D.*
Hoelen rubra	N.D.
Angelica gigas	N.D.
Trichosanthes kirilowii	N.D.
Schizopepon bryoniaefolius	N.D.
Arctium lappa (root)	N.D.
Gynostemma pentaphyllum	N.D.
Ulmus parvifolia (cortex)	N.D.
Finasteride	66.7 nM
Azelaic acid	0.0003%

(*: Not determined)

Table 2. K_i Values of Selected Natural Plants Extracts in Korea for Human Sebaceous Gland Cell 5-AR

Materials	Ki
Pinus thunbergii (leaf)	0.0011%
Oenothera odorata	0.0049%
Actinostemma lobatum	0.0086%
Finasteride	0.0006%
Azelaic acid	0.0011%

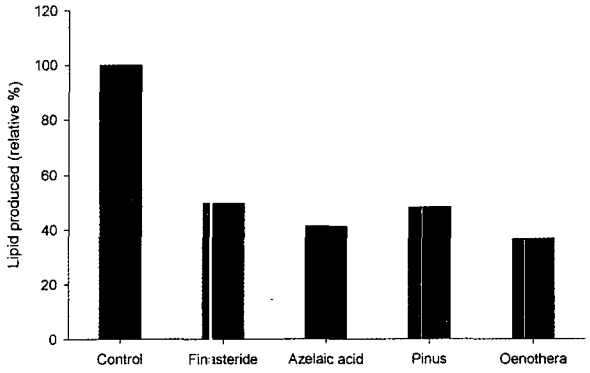

Figure 3. Sebosuppressive effects of selected natural extracts.

suring the total lipid quantity produced during co-incubation with the tested substances. Reduction rate of lipid production for *Pinus thunbergii* and *Oenothera odorata* was about 48% and 36%, respectively, which was comparable to that of finasteride or azelaic acid treated cells (Figure 3).

4. Conclusions

In the present study, we have tested the inhibitory effects of natural plant extracts on 5-AR from rat liver and human sebaceous gland derived cells. About eleven natural extracts showed an inhibitory activity on the rat liver microsomal 5-AR, and among the tested substances, *Pinus thunbergii* and evening primrose (*Oenothera odorata*) extracts showed potent 5-AR inhibitory activity. Since the rat liver microsomal 5-AR consists of two types of isozymes and its composition is significantly different from that of skin, we also tried to obtain type I isozyme-dominant 5-AR suspensions using human sebaceous gland derived cells. Inhibitory effects on 5-AR from human sebaceous gland derived cells were evaluated for selected plant extracts, and *Pinus thunbergii* extract showed the most potent activity, which is comparable to that of azelaic acid. Finally, sebosuppressive effects on human sebaceous gland cells were measured *in vitro*. Intercellular lipid quantity was reduced by all of the tested substances, and the reduction rate was about 35~50%, except *Actinostemma lobatum* extracts. As a conclusion, *Pinus thunbergii* extracts can be used as a new cosmeceutical agent to regulate sebum production and to alleviate the sebum-derived skin diseases.

References

1. G. S. Pilgram, van der J. Meulen, G. S. Gooris, H. K. Koerten, and J. A. Bouwstra, The influence of two azones and sebaceous gland lipids on the lateral organization of lipids isolated from human stratum corneum, *Biochim. Biophys. Acta*, **1511**, 244 (2001).
2. J. J. Thiele, S. U. Weber, and L. Packer, Sebaceous gland secretion is a major physiologic route of vitamin E delivered to skin, *J. Invest. Dermatol.*, **113**, 1006 (1999).
3. M. Marques, Y Pei, M. D. Southall, J. M. Johnston,

H. Arai, J. Aoki, T. Inoue, H. Seltmann, C. C. Zouboulis, and J. B. Travers, Identification of platelet-activating factor acetylhydrolase II in human skin, *J. Invest. Dermatol.*, **119**, 913 (2002).
4. L. Ge, J. S. Gordon, C. Hsuan, K. Stenn, and S. M. Proury, Identification of D-6 desaturase of human sebaseous gland: Expression and enzyme activity, *J. Invest. Dermatol.*, **120**, 707 (2003).
5. J. W. Fluhr, M. Man, B. E. Brown, *et al.*, Glycerol regulates stratum corneum hydration in sebaceous gland deficient (Asebia) mice, *J. Invest. Dermatol.*, **120**, 728 (2003).
6. P. E. Pochi and J. S. Strauss, Endocrinologic control of the development and activity of the human sebaceous gland, *J. Invest. Dermatol.*, **62**, 191 (1974).
7. Ch. C. Zouboulis, L. Xia, H. Akamatsu, H. Seltmann, M. Fritsch, S. Hornemann, R. Ruhl, W. Chen, H. Nau, and C. E. Orfanos, The human sebocyte culture model provides new insights into development and seborrhoea and acne, *Dermatol.*, **196**, 21 (1998).
8. D. Thiboutot, S. Jabara, J. M. McAllister, A. Sivarajah, K. Gilliland, Z. Cong, and G. Clawson, Human skin is a steroidogenic tissue: steroidogenic enzymes and cofactors are expressed in epidermis, normal sebocytes, and immobilized sebocyte cell line (SEB-1), *J. Invest. Dermatol.*, **120**, 905 (2003).
9. F. J. Ebling, E. Ebling, V. McCaffery, and J. Skinner, The response of the sebaceous gland of the hypophysectomised-castrated male rat to 5α-dihydrotestosterone, androstendiones, dehydroepiandrosterone and androsterone, *J. Endocrinol*, **51**, 181 (1971).
10. W. Chen, Ch. C. Zouboulis, and C. E. Orfanos, The 5α-reductase system and its inhibitors. *Dermatol.*, **193**, 177 (1996).
11. T. Fujie, T. Shikiji, N. Uchida, Y. Urano, H. Nagae, and S. Arase, Culture of cells derived from the human sebaceous gland under serum-free conditions without a biological feeder layer or specific matrices, *Arch. Dermatol. Res.*, **288**, 703 (1996).
12. Z. Sun, W. Zheng, J. Feng, and Z. Tu, A convenient and rapid method to study enzymatic kinetics of steroid 5α-reductase inhibitors, *Indian J. Pharmacol.*, **30**, 257 (1998).