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# Preventable effect of L-threonate, an ascorbate metabolite, on androgen-driven balding via repression of dihydrotestosterone-induced dickkopf-1 expression in human hair dermal papilla cells

*Mi Hee Kwack[1], Ji Sup Ahn[2], Moon Kyu Kim[1], Jung Chul Kim[1] & Young Kwan Sung[1,*]*

[1]Department of Immunology, School of Medicine, Kyungpook National University, Daegu 700-422, [2]Dr. Ahn Medical Hair Clinic, Seoul 135-996, Korea

**In a previous study, we recently claimed that dihydrotestosterone (DHT)-inducible dickkopf-1 (DKK-1) expression is one of the key factors involved in androgen-potentiated balding. We also demonstrated that L-ascorbic acid 2-phosphate (Asc 2-P) represses DHT-induced DKK-1 expression in cultured dermal papilla cells (DPCs). Here, we investigated whether or not L-threonate could attenuate DHT-induced DKK-1 expression. We observed via RT-PCR analysis and enzyme-linked immunosorbent assay that DHT-induced DKK-1 expression was attenuated in the presence of L-threonate. We also found that DHT-induced activation of DKK-1 promoter activity was significantly repressed by L-threonate. Moreover, a co-culture system featuring outer root sheath (ORS) keratinocytes and DPCs showed that DHT inhibited the growth of ORS cells, which was then significantly reversed by L-threonate. Collectively, these results indicate that L-threonate inhibited DKK-1 expression in DPCs and therefore is a good treatment for the prevention of androgen-driven balding. [BMB reports 2010; 43 (10): 688-692]**

## INTRODUCTION

The dermal papilla (DP) and dermal sheath of a mammalian hair follicle are derived from the mesenchyme. Hair follicles also contain epithelial cells in the outer root sheath (ORS), inner root sheath, matrix, and hair shaft that are derived from the epithelium (1). Reciprocal interactions between the epithelium and mesenchyme are essential for postnatal hair growth (2). The DP is known to play a key role in the regulation of hair growth and is encapsulated by the overlying follicular keratinocytes during hair growth period. Factors from the DP are believed to stimulate proliferation and differentiation of follicular keratinocytes into the hair shaft (3).

Male-pattern baldness (MPB) is the most common type of hair loss in men. Although its molecular pathogenic mechanism is not clear, dihydrotestosterone (DHT)-dependence has been well demonstrated in MPB (4, 5). Further, the treatment effects of finasteride, a selective inhibitor of type II 5α-reductase (5α-R II) that converts testosterone to DHT, support DHT-dependence in MPB (6). Circulating androgens such as DHT enter the follicle via capillaries in the DP, bind to androgen receptor (AR) within dermal papilla cells (DPCs), and then activate or repress target genes (7). Recent studies suggest that DHT-driven release of autocrine and paracrine factors from DPCs may be the key to androgen-potentiated balding (8-10).

We recently found that dickkopf 1 (DKK-1) is one of the most upregulated genes in balding DPCs (11). DKK-1 encodes a potent and specific endogenously-secreted Wnt antagonist that binds and inhibits low-density lipoprotein (LDL) receptor-related protein co-receptors that are involved in canonical Wnt signaling during hair induction and growth (12-14). Based on the finding that DHT-inducible DKK-1 expression in balding DPCs causes apoptosis in follicular keratinocytes, we claimed that DKK-1 is one of the key factors involved in androgen-potentiated balding (11).

L-ascorbic acid 2-phosphate (Asc 2-P) liberates L-ascorbic acid (AsA) via alkaline phosphatase present on the plasma membrane of various kinds of cells (15). This is followed by the incorporation of AsA into the cells. Very recently, we demonstrated that Asc 2-P represses DHT-induced DKK-1 expression in cultured DPCs of human hair follicles (16). In this study, we first investigated whether or not L-threonate, a metabolite of Asc 2-P, could attenuate DHT-induced DKK-1 expression in cultured DPCs by RT-PCR and ELISA. We next examined whether or not L-threonate could reverse the growth inhibitory role of DHT-inducible DKK-1 in follicular ORS kera-

*Corresponding author. Tel: 82-53-420-4874; Fax: 82-53-423-4628; E-mail: ysung@knu.ac.kr
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tinocytes using an *in vitro* co-culture system.

## RESULTS AND DISCUSSION

### L-threonate represses DHT-induced DKK-1 expression

Consistent with our previous report (11), we observed that 100 nM DHT induced DKK-1 mRNA expression by RT-PCR analysis (Fig. 1A, compare lanes 1 and 2). When L-threonate was added together with DHT, DHT-induced DKK-1 mRNA expression in DPCs was significantly attenuated (Fig. 1A, compare lanes 2, and 3 and 4). We next measured the concentration of DKK-1 in conditioned medium using ELISA. The mean concentration of DKK-1 was 11.49 ng/ml in the presence of 100 nM DHT and 5.25 ng/ml in the absence of DHT, demonstrating upregulation of DKK-1 in response to DHT (Fig. 1B, compare lanes 1 and 2). When 0.25 and 1 mM L-threonate was added together with DHT, the mean amount of DKK-1 was reduced to 5.03 and 5.62 ng/ml, respectively, demonstrating that DHT-induced DKK-1 secretion was repressed by L-threonate in DPCs (Fig. 1B, compare lanes 2, and 3 and 4).

### L-threonate represses DHT-induced activation of DKK-1 promoter activity

A pGL3-DKK-1 promoter plasmid that expresses a luciferase reporter gene at different levels in response to various levels of DKK-1 promoter activity was constructed and used to further confirm the repression of DHT-induced DKK-1 expression by L-threonate. We found that DKK-1 promoter activity was increased by DHT treatment (Fig. 2, compare lanes 1 and 2). When L-threonate was added together with DHT, DHT-induced activation of luciferase activity in DPCs was significantly repressed (Fig. 2, compare lanes 2, and 3 and 4).

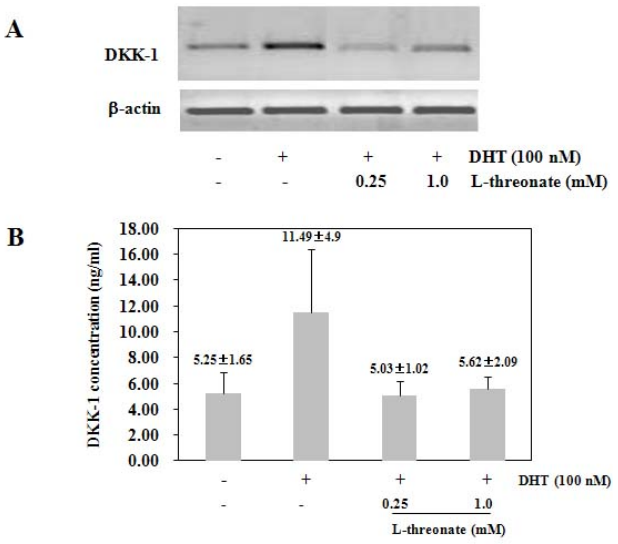


**Fig. 1.** Repression of DHT-induced DKK-1 expression by L-threonate. (A) Human dermal papilla cells (DPCs) were treated with 100 nM DHT for 6 h in the presence or absence of 0.25 and 1 mM L-threonate and analyzed by RT-PCR. (B) Human DPCs were treated with 100 nM DHT for four days in the presence or absence of 0.25 and 1 mM L-threonate, after which the concentrations of DKK-1 in conditioned medium were measured by ELISA. Data are expressed as means ± SD of three independent experiments.

### L-threonate attenuates DHT-induced growth inhibition of co-cultured keratinocytes

A co-culture system employing DPCs and keratinocytes has been used previously to analyze epithelial-mesenchymal inter-

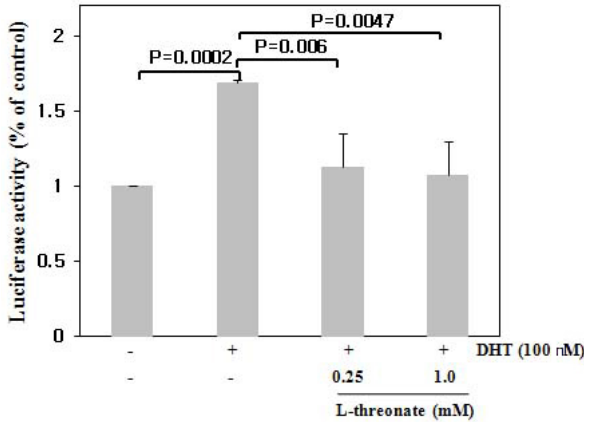


**Fig. 2.** Repression of DHT-induced activation of DKK-1 promoter by L-threonate. Human dermal papilla cells were treated with 100 nM DHT in the presence or absence of L-threonate, after which luciferase activity was measured. Data are expressed as means ± SD of three independent experiments.

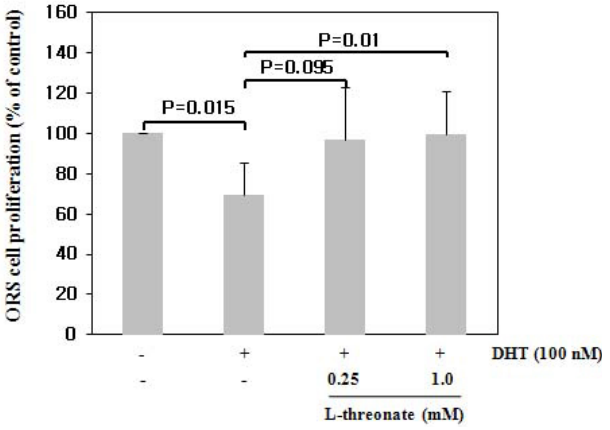


**Fig. 3.** Effect of L-threonate on DHT-mediated growth inhibition of ORS keratinocytes. Human dermal papilla cells and ORS keratinocytes were co-cultured in the absence (lane 1) or presence (lanes 2-4) of DHT for four days with 0.25 mM (lane 3) and 1 mM L-threonate (lane 4). Data are expressed as means ± SD of three determinations per experiment from three experiments.

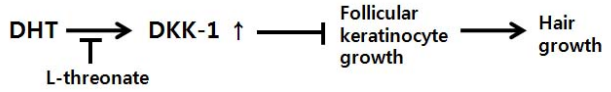


**Fig. 4.** Preventable effect of L-threonate on androgen-driven balding via repression of DHT-induced DKK-1 expression. This scheme is based on this study and previous reports (1-3, 11). The dermal papilla (DP) is encapsulated by overlying follicular keratinocytes during hair growth, with factors from the DP believed to cause proliferation and differentiation of follicular keratinocytes into the hair shaft (1-3). DHT-induced DKK-1 expression in dermal papilla cells was one of the key factors involved in androgen-driven balding (11). DHT-induced DKK-1 strongly inhibited follicular keratinocyte proliferation (11). L-threonate repressed DHT-induced DKK-1 expression in dermal papilla cells (this study).

actions stimulated by androgen via secreted soluble factors (8, 11, 17). We recently reported that DHT inhibits the growth of co-cultured ORS keratinocytes using this *in vitro* co-culture system and demonstrated that the growth suppression is largely due to DKK-1 (11). Consistent with this, the growth of co-cultured ORS cells was significantly suppressed in the presence of 100 nM DHT (Fig. 3, compare lanes 1 and 2). Since L-threonate repressed DHT-induced DKK-1 expression (Fig. 1 and 2), we investigated whether or not L-threonate attenuates DHT-induced growth suppression of co-cultured keratinocytes. We indeed observed that L-threonate reversed the DHT-induced growth inhibition of co-cultured keratinocytes (Fig. 3, compare lanes 2, and 3 and 4).

In summary, our data demonstrates that L-threonate repressed DHT-induced DKK-1 expression in cultured DPCs and attenuated DHT-induced growth inhibition of co-cultured keratinocytes. Although further investigation is needed to elucidate the mechanism of L-threonate-mediated repression of DHT-induced DKK-1 expression, our data in this study strongly suggest that L-threonate has an inhibitory effect on androgen-driven balding (Fig. 4).

## MATERIALS AND METHODS

### Culture of dermal papilla cells and keratinocytes

Punch biopsy (4 mm) specimens were taken from male scalps of patients undergoing hair transplantation surgery for androgenic alopecia. The medical ethical committee of Kyungpook National University Hospital (Korea) approved all studies and informed written consent was obtained from the patients. Hair follicles were isolated by a previously described method with minor modifications (18, 19). Briefly, subcutaneous fat portion of scalp skin including lower hair follicles was dissected from epidermis and dermis. Then, hair follicles were isolated under a binocular microscope using forceps.

Dermal papillae were isolated from the bulbs of dissected hair follicles, transferred onto plastic dishes coated with bovine type 1 collagen, and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 20% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 95% air/5% $CO_2$. The explants were left for several days, and the medium was changed every three days. After cell outgrowth had become sub-confluent, cells were harvested with 0.25% trypsin/10 mM EDTA in Hank's balanced salt solution (HBSS) and sub-cultured at a split ratio of 1 : 3. Afterwards, DP cells were maintained in DMEM supplemented with 10% FBS.

Keratinocytes were isolated from the same hair specimens. The hair shaft and hair bulb region of the hair follicle was cut off to prevent contamination with other cells. Trimmed hair follicles were immersed in DMEM-supplemented 20% FBS. On the third day of culture, the medium was changed to keratinocyte growth medium (KGM; Gibco BRL) containing penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (250 ng/ml). After subculture, keratinocytes were maintained in KGM, and cells from the second passage were used for further study.

### Co-culture of dermal papilla cells and keratinocytes

DP cells were cultured in serum-free DMEM and then plated at a density of $3 \times 10^4$ cells per well in the lower compartment of Transwell culture dishes (Costar, Cambridge, MA, USA). After 24 h, keratinocytes ($1.5 \times 10^4$ cells per well) were added to the upper compartment of the dish, which was separated from the lower compartment by a permeable membrane coated with type I and III collagen (pore size: 0.4 μm, 24-well format, Costar micro porous membrane filter). The two types of cells were then co-cultured in MCDB 153 medium (Sigma) without growth factors in the presence of DHT or vehicle as a control. In some experiments, L-threonate (L-threonic acid calcium salt; Sigma, St Louis, MO, USA) was added to the cultures. After four days, 3-[4,5]dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) was added (70 μg per well) for 3 h. The formazan produced was solubilized with DMSO, and optical density was measured at 570 nm.

### RT-PCR analysis

Total RNA was isolated using TRIzol reagent, and cDNA was synthesized from 3 μg of total RNA using an cDNA synthesis kit containing superscript II reverse transcriptase and oligo-dT primers according to the manufacturer's instructions (Promega, Madison, WI, USA). One microliter of cDNA was amplified with each of the forward and reverse primers. For the detection of DKK-1, 25 cycles (1 min at 94°C, 45 s at 58°C, and 45 s at 72°C) of amplification was performed with 5'-TGATGA GTACTGCGCTA GTC-3' and 5'-CTCCTATGCTTGGTACACAC-3'. For the detection of β-actin, 23 cycles (1 min at 94°C, 45 s at 58°C, and 45 s at 72°C) of amplification was performed with 5'-GGGAAATCGTGCGTGACATT-3' and 5'-GGAGTTGAAGG TAGTTTCGTG-3'. PCR products were separated by electrophoresis on 1% agarose gel and visualized under UV light.

### Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as previously described (20) with minor modifications. Briefly, Titer plates (Nunc-Immuno MaxiSorp surface) were coated with 100 μl of goat anti-human DKK-1 antibody (R&D Systems, Minneapolis, CA, USA) at a concentration of 1 μg/ml in PBS, incubated at 4°C overnight, and then blocked with 4% BSA at room temperature for 1 h. Culture-conditioned media (100 μl per well) was loaded and incubated at room temperature for 2 h, after which the wells were washed and incubated with biotinylated goat anti-human DKK-1 IgG (R&D Systems) diluted to a concentration of 0.2 μg/ml in dilution buffer and HRP-conjugated streptavidin (Pierce, Rockford, IL, USA.) for 30 min. TMB substrate solution (Pierce) was then added, and optical density was measured by an ELISA reader at 450 nm. Serial dilutions of recombinant human DKK-1 (R&D Systems) were used to establish a standard curve.

### Construction of human DKK-1 promoter/luciferase plasmid and reporter assay

Human genomic DNA was purified from cultured DP cells obtained from a patient with androgenetic alopecia using a QIAamp DNA Mini kit. A pGL3-DKK-1 promoter plasmid was constructed as described before (21). Briefly, the 988 bp upstream region of the human DKK-1 promoter was amplified by PCR using 5'-CTCACGCGTCTGCCTAATCA-3' (sense) and 5'-AAGCTTTCAGAAGGACTCAAG AGGGA-3' (antisense) as primers. After digestion with MluI and HindIII, the fragment was subcloned into the MluI/HindIII site of a promoterless luciferase expression vector, pGL3-Basic vector (Promega), which was designated as pGL3-DKK-1. Human DP cells ($10^5$ cells per well) were transiently transfected using a microporator (Disital Bio., Seoul, Korea) with 450 ng of pGL3-DKK-1 and 50 ng of pRLCMV (a Renilla luciferase vector, Promega) as an internal control. Twenty-four hours after transfection, the cells were incubated in serum-free DMEM with 100 nM DHT in the presence or absence of L-threonate. Firefly and Renilla luciferase activities were measured after 24 h using a dual luciferase assay kit (Promega).

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## REFERENCES

1. Hardy, M. H. (1992) The secret life of the hair follicle. *Trends Genet.* **8**, 55-61.
2. Botchkarev, V. A. and Kishimoto, J. (2003) Molecular control of epithelial-mesenchymal interactions during hair follicle cycling. *J. Investig. Dermatol. Symp. Proc.* **8**, 46-55.
3. Stenn, K. S. and Paus, R. (2001) Controls of hair follicle cycling. *Physiol. Rev.* **81**, 449-494.
4. Imperato-McGinley, J., Guerrero, L., Gautier, T. and Peterson, R. E. (1974) Steroid 5alpha-reductase deficiency in man: an inherited form of male pseudohermaphroditism. *Science* **186**, 1213-1215.
5. Kuttenn, F., Mowszowicz, I., Wright, F., Baudot, N. and Mauvais-Jarvis, P. (1979) Male pseudohermaphroditism: a comparative study of one patient with 5 alpha-reductase deficiency and three patients with the complete form of testicular feminization. *J. Clin. Endocrinol. Metab.* **49**, 861-865.
6. Drake, L., Hordinsky, M., Fiedler, V., Swinehart, J., Unger, W. P. and Waldstreicher, J. (1999) The effects of finasteride on scalp skin and serum androgen levels in men with androgenetic alopecia. *J. Am. Acad. Dermatol.* **41**, 550-554.
7. Randall, V. A., Thornton, M. J., Hamada, K. and Messenger, A. G. (1994) Androgen action in cultured dermal papilla cells from human hair follicles. *Skin Pharmacol.* **7**, 20-26.
8. Inui, S., Fukuzato, Y., Nakajima, T., Yoshikawa, K. and Itami, S. (2002) Androgen-inducible TGF-beta1 from balding dermal papilla cells inhibits epithelial cell growth: a clue to understand paradoxical effects of androgen on human hair growth. *FASEB J.* **16**, 1967-1969.
9. Hibino, T. and Nishiyama, T. (2004) Role of TGF-beta2 in the human hair cycle. *J. Dermatol. Sci.* **35**, 9-18.
10. Hamada, K. and Randall, V. A. (2006) Inhibitory autocrine factors produced by the mesenchyme-derived hair follicle dermal papilla may be a key to male pattern baldness. *Br. J. Dermatol.* **154**, 609-618.
11. Kwack, M. H., Sung, Y. K., Chung, E. J., Im, S. U., Ahn, J. S., Kim, M. K. and Kim, J. C. (2008) Dihydrotestosterone-inducible dickkopf 1 from balding dermal papilla cells causes apoptosis in follicular keratinocytes. *J. Invest. Dermatol.* **128**, 262-269.
12. Millar, S. E., Willert, K., Salinas, P. C., Roelink, H., Nusse, R. and Barsh, G. S. (1999) WNT signaling in the control of hair growth and structure. *Dev. Biol.* **207**, 133-149.
13. Kishimoto, J., Burgeson, R. E. and Morgan, B. A. (2000) Wnt signaling maintains the hair-inducing activity of the dermal papilla. *Genes. Dev.* **14**, 1181-1185.
14. Shimizu, H. and Morgan, B. A. (2004) Wnt signaling through the beta-catenin pathway is sufficient to maintain, but not restore, anagen-phase characteristics of dermal papilla cells. *J. Invest. Dermatol.* **122**, 239-245.
15. Takamizawa, S., Maehata, Y., Imai, K., Senoo, H., Sato, S. and Hatta, R. (2004) Effects of ascorbic acid and ascorbic acid 2-phosphate, a long-acting vitamin C derivative, on the proliferation and differentiation of human osteoblast-like cells. *Cell Biol. Int.* **28**, 255-265.
16. Kwack, M. H., Kim, M. K., Kim, J. C. and Sung, Y. K. (2010) L-Ascorbic acid 2 phosphate represses the dihydrotestosterone-induced dickkopf-1 expression in human balding dermal papilla cells. *Exp. Dermatol.* (In press), DOI:10.1111/j.1600-0625.2010.01143.x.
17. Itami, S., Kurata, S. and Takayasu, S. (1995) Androgen in-

duction of follicular epithelial cell growth is mediated via insulin-like growth factor-1 from dermal papilla cells. *Biochem. Biophys. Res. Commun.* **212**, 988-994.
18. Philpott, M. P., Sanders, D. A., Westgate, G. E. and Kealey, T. (1994) Effects of insulin and insulin-like growth factors on cultured human hair follicles: IGF-1 at physiologic concentrations is an important regulator of hair follicle. *J. Invest. Dermatol.* **102**, 857-861.
19. Magerl, M., Kauser, S., Paus, R. and Tobin, D. J. (2002) Simple and rapid method to isolate and culture follicular papillae from human scalp hair follicles. *Exp. Dermatol.* **11**, 381-385.
20. Tian, E., Zhan, F., Walker, R., Rasmussen, E., Ma, Y. and Shaughnessy, J. D. (2003) The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. *N. Engl. J. Med.* **349**, 2483-2494.
21. Ohnaka, K., Taniguchi, H., Kawate, H., Nawata, H. and Takayanagi, R. (2004) Glucocorticoid enhances the expression of dickkopf-1 in human osteoblast: novel mechanism of glucocorticoid-induced osteoporosis. *BBRC* **318**, 259-264.