Journal of Dermatological Science 72 (2013) 16–24

Contents lists available at SciVerse ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Valproic acid promotes human hair growth in *in vitro* culture model

Seong Jin Jo [a,b,c], Soon-Jin Choi [a], Sun-Young Yoon [a], Ji Yeon Lee [a], Won-Seok Park [d], Phil-June Park [d], Kyu Han Kim [a,b,c], Hee Chul Eun [a,b,c,1,*], Ohsang Kwon [a,b,c,1,**]

[a] *Department of Dermatology, Seoul National University College of Medicine, Seoul, Republic of Korea*
[b] *Institute of Human–Environment Interface Biology, Seoul National University, Seoul, Republic of Korea*
[c] *Laboratory of Cutaneous Aging and Hair Research, Biomedical Research Institute, Seoul National University Hospital, Seoul, Republic of Korea*
[d] *Advanced Hair Research Laboratory, AmorePacific R&D Center, Republic of Korea*

ARTICLE INFO

Article history:
Received 26 September 2012
Received in revised form 16 May 2013
Accepted 20 May 2013

Keywords:
Valproic acid
GSK-3 β
β-catenin
Hair
Dermal papilla

ABSTRACT

Background: β-Catenin, the transducer of Wnt signaling, is critical for the development and growth of hair follicles. In the absence of Wnt signals, cytoplasmic β-catenin is phosphorylated by glycogen synthase kinase (GSK)-3 and then degraded. Therefore, inhibition of GSK-3 may enhance hair growth *via* β-catenin stabilization. Valproic acid is an anticonvulsant and a mood-stabilizing drug that has been used for decades. Recently, valproic acid was reported to inhibit GSK-3β in neuronal cells, but its effect on human hair follicles remains unknown.
Objectives: To determine the effect of VPA on human hair growth.
Methods: We investigated the effect of VPA on cultured human dermal papilla cells and outer root sheath cells and on an *in vitro* culture of human hair follicles, which were obtained from scalp skin samples of healthy volunteers. Anagen induction by valproic acid was evaluated using C57BL/6 mice model.
Results: Valproic acid not only enhanced the viability of human dermal papilla cells and outer root sheath cells but also promoted elongation of the hair shaft and reduced catagen transition of human hair follicles in organ culture model. Valproic acid treatment of human dermal papilla cells led to increased β-catenin levels and nuclear accumulation and inhibition of GSK-3β by phosphorylation. In addition, valproic acid treatment accelerated the induction of anagen hair in 7-week-old female C57BL/6 mice.
Conclusions: Valproic acid enhanced human hair growth by increasing β-catenin and therefore may serve as an alternative therapeutic option for alopecia.

1. Introduction

A hair follicle (HF) is an organ composed of epithelial and mesenchymal tissues. It grows in a unique and cyclic fashion through 3 phases: anagen phase of growth, catagen phase of involution, and telogen phase of rest [1]. The interaction between epithelial and mesenchymal tissues is vital for the growth and development of HFs [2,3]. The dermal papilla (DP), a cluster of mesenchymal cells, is enveloped by hair matrix keratinocytes in the bulb of anagen hair and activates them to maintain and regenerate the hair growth cycle [4]. Moreover, effective interaction between the DP and surrounding epithelial cells is essential to form new HFs [5]. Therefore, DP plays a decisive role in the regulation of hair growth and reconstitution of HFs.

Numerous molecular signals orchestrate hair growth, and the Wnt/β-catenin signal is one of the most important of these molecular signals. Previous studies have shown that β-catenin in DP cells (DPCs) is markedly activated during anagen phase [6,7] and is important for the maintenance of anagen phase and regeneration of the hair cycle [8,9]. Recently, Enshell-Seijffers et al. reported on the premature induction of catagen and telogen phase and the failure of regeneration of the cycling follicle in β-catenin knock-out mice [10]. These studies suggest that activation of β-catenin in DP can prolong the anagen phase and promote hair growth.

β-Catenin protein levels are regulated by the Wnt signal. In the absence of Wnt, β-catenin is ordinarily phosphorylated in the cytoplasm by glycogen synthase kinase (GSK)-3, a serine/threonine protein kinase encoded by *GSK-3* α and β. Phosphorylated β-catenin is tagged by a small protein ubiquitin and then destroyed by the proteasome. However, when Wnt protein binds to cell

* Corresponding author at: Department of Dermatology, Seoul National University College of Medicine, 101 Daehak-ro, Chongno-Gu, Seoul 110-744, Republic of Korea. Tel.: +82 2 2072 2415; fax: +82 2 742 7344.
** Corresponding author at: Department of Dermatology, Seoul National University College of Medicine, 101 Daehak-ro, Chongno-Gu, Seoul 110-744, Republic of Korea. Tel.: +82 2 2072 1996; fax: +82 2 742 7344.
E-mail addresses: hceun@snu.ac.kr (H.C. Eun), oskwon@snu.ac.kr (O. Kwon).
[1] These authors contributed equally to this work.

0923-1811/$36.00
http://dx.doi.org/10.1016/j.jdermsci.2013.05.007

surface receptors of the Frizzled family, β-catenin destruction is prevented by the inhibition of GSK-3. As a result, β-catenin accumulates in the cytoplasm and moves into the nucleus, where it activates gene expression programs [11]. Thus, GSK-3 is a key protein in the regulation of Wnt/β-catenin signaling, and inhibition of GSK-3 may enhance hair growth through an increase in β-catenin levels in DPCs. However, clinical therapies targeting Wnt/β-catenin signaling have not yet been developed.

Valproic acid (VPA) is the most widely used anticonvulsant since its approval for epilepsy in 1967 [12]. Although its mechanism of action as an anticonvulsant is still under investigation, inhibition of GSK-3β in neuronal cells is considered as one of the possible antiepileptic mechanisms of VPA [11,13,14]. Thus, considering the importance of Wnt/β-catenin signaling in hair growth, treatment of hair with VPA may promote hair growth *via* inhibition of GSK-3β in HFs, similar to that in neuronal cells. However, the effect of VPA on GSK-3β seems to depend on cell type. Although many studies have reported that VPA directly inhibits GSK-3β in human neuroblastoma SH-SY5Y and human hepatocarcinoma HepG2 cells [15–17], other studies with murine Neuro2A neuroblastoma cells and rat primary cultured neurons have failed to show the inhibition of GSK-3β by VPA [18,19]. Because of this inconsistency, the effect of VPA on hair growth could not be anticipated before this study.

This study aimed to determine the effect of VPA on human hair growth. For this, we investigated the effect of VPA on cultured human DPCs (hDPCs) and outer root sheath (ORS) cells, on an *in vitro* culture of human HFs, and on anagen hair induction in C57BL/6 mice. VPA enhanced the proliferation of cultured hDPCs and ORS cells; moreover, hair shafts showed more rapid elongation when HFs were cultured *in vitro* with VPA. In addition, anagen hair induction in mice was promoted by topical VPA treatment.

2. Materials and methods

2.1. Ethics statement

Study protocols were approved by the institutional research board of Seoul National University Hospital (H-1002-046-309), and written informed consent was obtained from all subjects. All experimental procedures using human tissues were performed according to the principles described in the declaration of Helsinki.

2.2. Human hair follicle samples

Healthy male volunteers (age, 20–40 years) without current or prior scalp diseases provided 1.5 × 1.0 cm scalp tissue samples from their occipital region. The samples were carefully dissected into single HFs under a stereomicroscope (Olympus, Tokyo, Japan). In this study, only anagen HFs were used, as described previously [20].

2.3. Cell culture

Human DPCs were cultured as described previously [21]. Briefly, candlelight-shaped DP was dissociated and incubated in Dulbecco's modified Eagle medium (Welgene, Daegu, Republic of Korea) supplemented with 10% fetal bovine serum (Welgene) and antibiotics (100 mg/mL streptomycin and 100 U/mL penicillin) at 37 °C in a 5% CO_2 atmosphere.

For ORS cells, the lower follicle between the sebaceous duct and bulb was incubated in keratinocyte growth medium (Clonetics, San Diego, CA, USA) containing MCDB153 medium supplemented with epidermal growth factor (10 ng/mL), bovine pituitary extract (70 g/mL), hydrocortisone (0.5 g/mL), insulin (5 g/mL), penicillin (100 g/mL), and fungizone (0.25 g/mL) at 37 °C in a 5% CO_2 atmosphere.

2.4. Thiazolylblue (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) (MTT) assay

Cell viability was measured by the MTT assay as previously described [22]. Cells at 5.0×10^3 cells/200 μL per well were seeded into 96-well plates, serum-starved for 24 h, and then treated with VPA (Sigma–Aldrich, St. Louis, MO, USA) for 4 days. After adding 20 μL of MTT solution (5 mg/mL), cells were incubated for 4 h at 37 °C in the dark and then incubated with 200 μLof dimethyl sulfoxide for 20 min at room temperature. The samples were assessed by measuring the absorbance at 570 nm with an ELISA reader, and the results were expressed as fold changes relative to the negative control.

2.5. Human hair follicles organ culture and immunofluorescence staining

Isolated human scalp hair follicles were cultured as described previously [23]. Briefly, each hair follicle was cut at the level of the sebaceous duct and cultured in Williams E medium (Gibco BRL, Gaithersburg, MD, USA) containing 10 ng/mL hydrocortisone, 10 μg/mL insulin, 2mM L-glutamine, and 100 U/mL penicillin at 37 °C in a 5% CO_2 atmosphere for 12 days. VPA was added at final concentrations of 0.01, 0.1, and 1.0 mM. At every third day, elongation of the hair shaft was measured, and HFs in anagen phase were determined according to their growth.

Immunofluorescence staining was performed to evaluate proliferation and apoptosis in HF matrix keratinocytes. As previously described [24], immunoreactivity for Ki67 (DAKO, Carpinteria, CA, USA) and TUNEL labeling (*in situ* cell death detection kit,fluorescein, Roche, Diagnostics, Mannheim, Germany) were used as an indicator of cell proliferation and apoptosis, respectively. A DAPI mounting media kit (Vector Laboratories, Burlingame, CA, USA) was used to counterstain the nuclei.

2.6. Western blot analysis

Soluble proteins were extracted using RIPA lysis buffer (Millipore, Billerica, MA, USA). The monoclonal antibodies were as follows: anti-β-actin antibody (SC-1616, Santa Cruz biotechnology, Santa Cruz, CA, USA), anti-β-catenin antibody (#9562, Cell Signaling Technology, Danvers, MA, USA), anti-dephospho-β-catenin antibody (ALX-804-259, Enzo Life Science, Plymouth Meeting, PA, USA), anti-GSK-3β antibody (#9315, Cell Signaling Technology), and anti-phospho-GSK-3β(Ser^9) antibody (#9336, Cell Signaling Technology).

2.7. Immunocytochemistry assay

Human DPCs at 1.0×10^4 cells/500 μL per chamber were seeded into a chamber slide, serum-starved for 24 h, and then treated with VPA for 48 h. After treatment with 4% paraformaldehyde for 10 min and 0.1% triton X-100 for 5 min, the cultured hDPCs were incubated with anti-β-catenin antibody at 4 °C overnight and then with Alexa Fluor 594-labeled goat anti-mouse IgG (Invitrogen Japan). A DAPI mounting media kit was used to counterstain the nuclei.

2.8. Quantitative real-time polymerase chain reaction

Gene expressions were measured as described previously [24], with the primers listed in Supplementary Table 1. Data were analyzed using the $2^{-\Delta\Delta CT}$ method and presented as the fold change relative to the control normalized to 36B4 expression. The experiment was performed in triplicate and repeated at least 4 times.