Hair Growth Promoting Potential of Phospholipids Purified from Porcine Lung Tissues

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Abstract

BP201, porcine lung tissue-derived phospholipids, consists of phosphatidylcholine as a major phospholipid species. BP201 promoted hair growth after application onto the shaved backs of BALB/c and C3H mice. Its effect was enhanced when applied together with minoxidil (MNX) in C3H mice. When the tissue specimens prepared from the shaved skins of BP201-treated and control mice were microscopically examined, the total numbers of hair follicles in both anagen and telogen phases of BP201-treated mice were significantly higher than those of control mice. The numbers of hair follicles in the anagen phase of BP201-treated mice were also higher than those of control mice. In combination with MNX, BP201 further increased the total number of hair follicles, but did not alter the percentage of hair follicles in the anagenic phase. BP201 also increased the proliferation of human hair follicle dermal papilla cells. Collectively, BP201 possesses hair growth promoting potential, which would suggest its use singly or in combination for hair growth products.

Key Words: Phospholipid, Hair growth-promoting effect, Hair follicle, Dermal papilla cell

INTRODUCTION

Healthy hair is maintained via a cyclic process involving the regeneration of hair follicles in a stem cell-dependent manner. The hair cycle is comprised of three main phases, including a finite period of hair growth (anagen phase), a brief regression phase (categen phase), and a resting period (telogen phase) (Hoffmann and Happle, 2000). It is regulated by sensory neurons, cytokines, growth factors (GFs), and androgens, such as testosterone and dihydrotestosterone (Paus and Cotsarelis, 1999; Stenn and Paus, 2001). The growth of new hair requires reentry into the anagen phase, which involves the activation of multipotent epithelial stem cells residing in the outer hair root sheath (Taylor *et al*., 2000).

Hair loss is associated with the progressive miniaturization of the hair follicle and alteration of the hair cycle (Courtois *et al*., 1994). According to a recent report, 30% of males over 30 years of age and more than 50% of males over 50 years of age are affected by alopecia (Roy *et al*., 2008). Although female clinical signs are usually milder and associated with diffuse thinning of the scalp hair, women may also suffer from alopecia areata, defined as one type of hair loss that typically causes patches of baldness (Yoon *et al*., 2010).

Phosphatidic acid possessed potent growth effects on murine hair epithelial cells and epidermal keratinocytes, by promoting the anagen phase of the hair cycle in C3H mice (Takahashi *et al*., 2003). This hair growth-promoting effect was mediated by activation of the mitogen-activated protein (MAP) kinase/extracellular signal-regulated kinase (ERK) kinase pathway and its protective action on transforming growth factor (TGF)-β1-induced apoptosis (Takahashi *et al*., 2003). Ascorbic acid 2-phosphate promoted hair growth by inducing early telogen-to-anagen phase conversion, promoting dermal papilla cell (DPC) growth, and elongating shafts in hair follicles (Sung *et al*., 2006). Cyclosporin A exerted hair-growing effects by downregulating negative hair-growing factors in hair epithelial cells (Harmon *et al*., 1995; Takahashi and Kamimura, 2001).

Essential oils from the seeds of *Zizyphus jujube* increased the number of hair follicles in the anagen phase, as well as the hair length, weight, and thickness (Yoon *et al*., 2010). Essential oils from *Chamaecyparis obtuse* promoted the early phase

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of murine hair growth through induction of vascular endothelial growth factor (VEGF), a positive regulator of hair growth (Lee *et al*., 2010). Testosterone 5α-reductase activity was inhibited by an aqueous ethanol extract prepared from spores of *Lygodium japonicum*, the inhibitory factors of which are oleic, linoleic, and palmitic acids (Matsuda *et al*., 2002). Similarly, an acetone extract of *Boehmeria nipononivea*, which contains α-linolenic, elaidic, and stearic acids, displayed testosterone 5α-reductase inhibitory activities and hair growth-promoting effects in mice (Shimizu *et al*., 2000). Bovine milk polar lipids (including phospholipids) induced earlier progression of the hair cycle in mice, to an extent comparable to that of minoxidil (MNX), a well-recognized initiator of the anagen phase (Kumura *et al*., 2012). In addition to lipid components, several pure substances and mixtures have been also shown to exert hair growth-promoting effects through diverse mechanisms. Leaf extracts of *Hibiscus rosasinensis* Linn. showed strong hair-growth potential in *in vivo* and *in vitro* alopecia models (Adhirajan *et al*., 2003). The methanol extract of *Eclipta alba* promoted hair growth by inducing anagen in telogen phase hair follicles (Roy *et al*., 2008; Datta *et al*., 2009). Placenta extract is used in Chinese folk medicines to accelerate wound healing, as this extract contains abundant GFs and hormones, such as VEGF, TGF-β, epidermal growth factor (EGF), and insulin-like growth factor-1 (IGF-1) (Zhang *et al*., 2011). Placenta extract accelerated proliferation of hair follicle and interfollicle cells by preventing hair follicles from entering into a categen-like state (Zhang *et al*., 2011). Experiments in C57BL/6 and C3H mice revealed the hair growth-promoting effect of *Asiasari radix* extract, which enhanced protein synthesis in vibrissae follicle cultures and the proliferation of human keratinocytes and DPCs (Rho *et al*., 2005). An extract of dried *Sophora flavescens* root induced earlier telogen-to-anagen phase conversion in C57BL/6 mice by regulating GFs in DPCs. This extract also inhibited type II 5α-reductase catalyzing the formation of dihydrotestosterone from testosterone (Roh *et al*., 2002).

Until recently, purified phospholipid mixtures and individual phospholipid species, excluding phosphatidic acid, had not been tested for their hair growth-promoting effects. BP201 is a phospholipid mixture purified from porcine lung tissues and is being developed as a remedy for atopic dermatitis (Moon *et al*., 2012). Its principal component is phosphatidylcholine, of which 1,2-dipalmitoylphosphatidylcholine is predominant (Moon *et al*., 2012). In previous studies, BP201 was shown to alleviate symptoms of 2,4-dinitrofluorobenzene-induced allergic contact dermatitis in mice (Moon *et al*., 2012). BP201 has anti-inflammatory and related antinociceptive and anti-angiogenic activities, which indirectly support its use as an anti-atopic dermatitis therapy (Jung *et al*., 2012). Indeed, the phase III clinical trial for use of BP201 as an anti-atopic dermatitis treatment has been completed, and a BP201-containing product is expected to be commercially available in the near future.

Upon examining the effects of BP201 on induced allergic contact dermatitis in BALB/c mice, it was observed that BP201 could promote hair growth over shaved areas on the mouse backs. This finding urged us to test the hair growth-promoting effects of BP201. In the present study, we demonstrate that BP201 possesses potent hair growth-promoting effects.

MATERIALS AND METHODS

Drugs, reagents and porcine lung tissues

5% Minoxidil (MNX) solution was obtained from Hyundai Pharmaceutical Co. (Seoul, Korea). 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Penicillin-streptomycin was from Gibco-BRL (Gaithersburg, MD, USA). Detach kit was purchased from PromoCell (Heidelberg, Germany). Fresh porcine lung tissues were obtained from a slaughterhouse in Hongchun, Kangwon-do, Korea. The slaughterhouse met the Hazard Analysis Critical Control Point criteria. Tissues were immediately placed in an ice-cold water bath, and were used within 5 h of animal sacrifice.

Mice

Eight-week-old male BALB/c mice (Daehan Biolink, Korea) (27.0 ± 1.4 g) and six-week-old male C3H/He mice (Central Laboratory Animal, Korea) (25.9 ± 1.8 g) whose hair cycle was in the telogen phase were used in this study. The animal holding room was maintained at 25 ± 2°C under a 12-h light/dark cycle. Food and tap water were supplied *ad libitum*. All animal experiments performed were approved by the Ethical Committee of Kangwon National University, Korea.

Cell culture

Human hair follicle dermal papilla cells (HFDPCs) (Promo-Cell, Germany) were grown in DPC growth medium (Promo-Cell, Germany) containing 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL, MD) in 5% CO_2 at 37°C.

Purification of BP201

BP201 was purified from fresh porcine lung tissues, as described previously (Moon *et al*., 2012). Briefly, porcine lung tissues were diced and homogenized in three volumes of ice-cold saline containing 5 mM $CaCl_2$ in a metal-blade blender (Hanil Electric, Korea) for 1 min. Homogenized tissues were incubated in an ice-cold water bath for 1 h, and then centrifuged at 5,000 *g* for 45 min at 4°C. The precipitate was resuspended in six volumes of hypotonic 70 mM NaCl solution containing 5 mM $CaCl_2$, and homogenized in a metal-blade blender for 1 min. The homogenate was incubated in ice-cold water for 1 h and centrifuged at 5,000 *g* for 45 min at 4°C. Calcium ions were added to induce the aggregation of surfactant lipids and proteins in homogenates, to facilitate their precipitation at low centrifugal force. The final pellets were dissolved in two volumes of a chloroform:methanol (2:1 by volume) mixture at room temperature. Aqueous-phase and organic solvent-insoluble materials were discarded.

To eliminate neutral lipids from the organic solvent phase, the chloroform/methanol mixture was thoroughly evaporated in a rotary vacuum evaporator (Tokyo Rikakikai, Japan) at 65°C. Excess cold acetone was poured into the dried lipid mixture. The pool of polar lipids containing phospholipids was recovered as the precipitate, as they are insoluble in acetone. This polar lipid mixture, dissolved in pure chloroform, was further purified in an open silica gel column (Merck, Darmstadt, Germany), prewashed, and saturated with pure acetone.

To remove neutral lipids from the pool of polar lipids, non-polar solvents, such as acetone and chloroform, were added to the column. A polar solvent mixture of chloroform, methanol, and water was used later to obtain the pure phospholipids.

Eluting solvents were applied to the column in the following order: one bed volume of pure acetone, one bed volume of pure chloroform, one-third bed volume of a chloroform:methanol (9:1 by volume) mixture, one-third bed volume of a chloroform: methanol (4:1 by volume) mixture, and five or six bed volumes of a chloroform:methanol:water (6:8:1 by volume) mixture.

Among the fractions eluted by the final solution, fractions showing Rf values between 0.30 and 0.55 on the thin-layer chromatography developed by the chloroform: methanol:water (65:25:4 by volume) mixture were collected. The collected fractions were concentrated in a rotary vacuum evaporator, to remove solvents and moisture. Fractions were freeze-dried for 20 h at -98°C (Operon, Korea), to create the white powder of a phospholipid mixture, BP201. Finally, BP201 was suspended in distilled water, and then stored for months at -20°C.

Hair growth activity *in vivo*

Hair growth activity in mice was determined visually according to a slight modification of the method described by Hattori and Ogawa (Hattori and Ogawa, 1983). After the mice were acclimatized to their housing environment for 7 days, a dorsal portion of hair measuring approximately 4 cm^2 was shaved with electric hair clippers.

BALB/c mice were divided into two groups of five mice each: a control group and a BP201 experimental group. Distilled water was topically applied to the shaved skins of mice in the control group. Then, 60 μL of 2% BP201 solution were applied to the BP201 group. Distilled water or BP201 solution was gently rubbed until it is completely absorbed into the skin.

C3H mice were divided into four groups of five mice each, according to the samples that were topically applied: distilled water (control group), 2% BP201 (BP201 group), 5% MNX (Hyundai Pharmaceutical, Korea) (positive control group), and 2% BP201+5% MNX (combination treatment group). For all groups, 60-μl samples were applied twice daily for 21 days. Mice were sacrificed by CO_2 exposure on the 21st day. Their backs were then shaved, visually evaluated, and photographed.

Determination of hair growth area

Hair regrowth and nongrowth areas were photographed and printed. Regrowth and nongrowth regions were cut from the prints. Mice were weighed with an electronic balance. The percentages of hair regrowth areas relative to total shaved skin areas were calculated as follows: Hair growth area (%)=100×[regrowth area weight/(regrowth area weight+nongrowth area weight)].

Hair length determination

The average hair length on the shaved area was determined as previously described (Yoon *et al*., 2010). Briefly, after 21 days, the mice were sacrificed by CO_2 exposure. Hairs were plucked randomly from the shaved area. The lengths of 20 hairs were measured, and the average length was calculated.

Histological analysis

Dorsal skin biopsies were taken from C3H mice after treatment with BP201 and/or MNX. Biopsies were fixed in a 10% formaldehyde solution, embedded in paraffin, and sectioned for hematoxylin and eosin staining. The number of hair follicles in each shaved skin area and the ratio of hair follicles in ana-
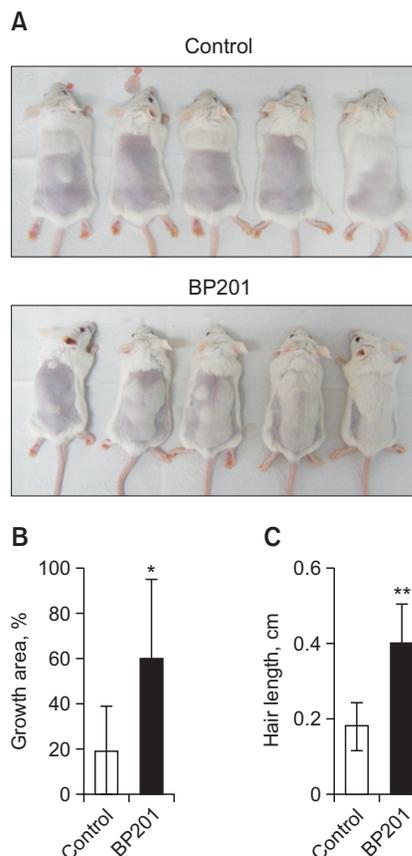

Fig. 1. Hair growth-promoting effect of BP201 in BALB/c mice. A 60-μL sample of distilled water (control) or 2% BP201 was topically applied to a shaved area on the back of a BALB/c mouse twice daily for 21 days. Mice were sacrificed, and their dorsal surfaces were photographed (A). The growth area (%) (B) and average hair length on the shaved area (C) were determined. Each value represents the mean ± SD of five mice. *$p<0.05$; **$p<0.01$ compared to the control group.

gen and telogen phases were determined by light microscopy.

Cell proliferation assay

Proliferation of HFDPCs in the presence of BP201 was determined by an MTT assay used to assess metabolic activity (Yang *et al*., 1999). A total of $5×10^4$ cells were seeded into each well of a 24-well plate, and treated with varying concentrations of BP201 for 72 h. The treated cells were incubated with 50 μL of 5 mg/mL MTT solution for 1 h. After removing the medium by suction, the cells were lysed with 300 μL isopropyl alcohol, and the amount of formazan, produced from reduction of MTT by the mitochondria of living cells, were determined by the absorbance at 540 nm.

Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Statistical comparisons between experimental groups were performed with unpaired Student's *t*-tests. *p*-values of less than 0.05 were considered significant.
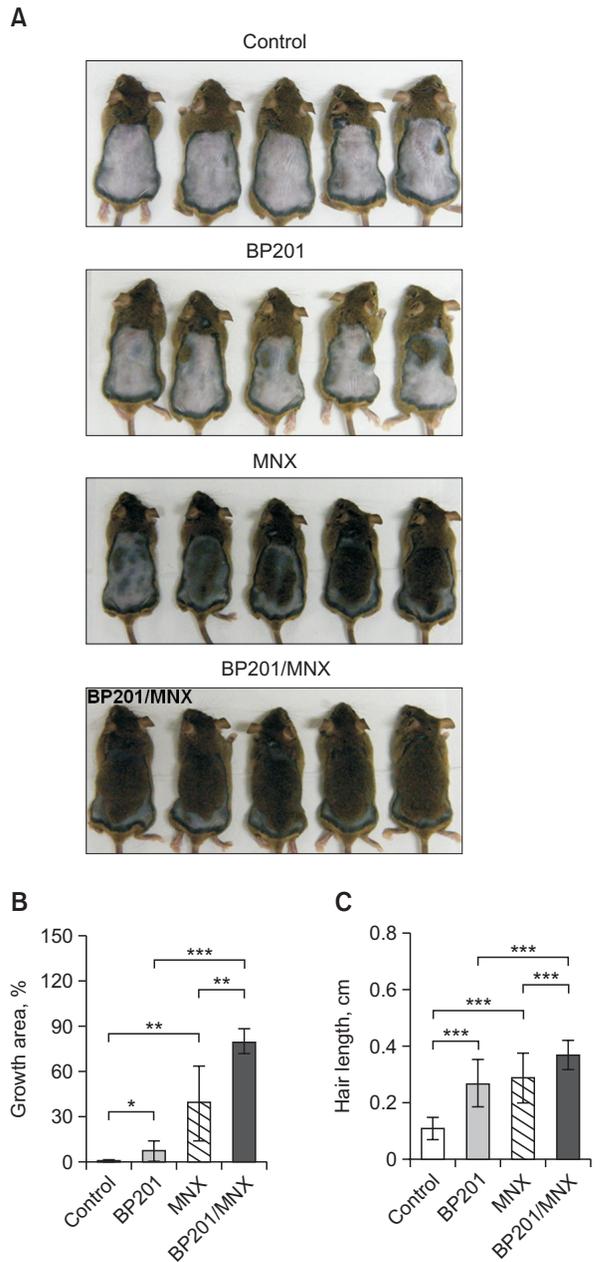
A

B

Fig. 3. Effects of BP201 on the total numbers of hair follicles in telogen and anagen phases (A) and on the percentage of anagen hair follicles (B) in C3H mice. A 60-μL sample of distilled water (control), 2% BP201 (BP201), 5% minoxidil (MNX), or 2% BP201 +5% minoxidil (BP201/MNX) was topically applied to the shaved areas of C3H mice twice daily for 21 days. Mice were sacrificed, and dorsal skin biopsies were analyzed. Each value represents the mean ± SD of five mice. *p<0.05; ***p<0.001 compared to the control group.

A

Control

BP201

MNX

BP201/MNX

B

C

Fig. 2. Hair growth-promoting effect of BP201 in C3H mice. A 60-μL sample of distilled water (control), 2% BP201 (BP201), 5% minoxidil (MNX) or 2% BP201 + 5% minoxidil (BP201/MNX) was topically applied to the shaved backs of C3H mice twice daily for 21 days. Mice were sacrificed, and their dorsal surfaces were photographed (A). Growth area (%) (B) and average hair length on the shaved area (C) were determined. Each value represents the mean ± SD of five mice. *p<0.05; **p<0.01; ***p<0.001 compared to the corresponding reference group.

RESULTS

Hair growth promoting effect of BP201 in BALB/c mice

The plausible hair growth promoting activity of BP201 was incidentally observed in our previous work employing BALB/c mouse as an animal model of contact dermatitis (Jung *et al*.,
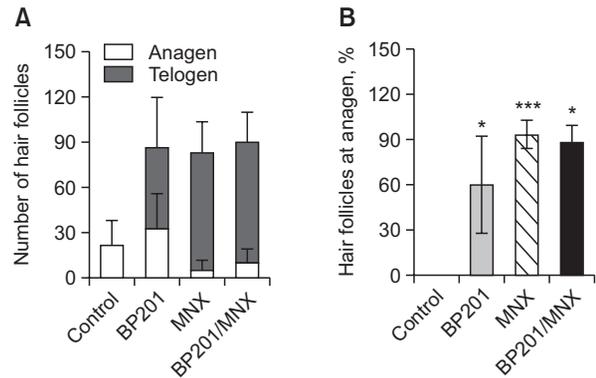
2012). To revalidate this possibility, the hair growth promoting effect of BP201 was systematically tested using BALB/c mouse. Hair growth on the shaved skins of mice in the BP201-treated group was enhanced compared to the control group (Fig. 1A). The hair growth area in control mice was 19.2%, compared to 60.0% in BP201-treated mice (Fig. 1B). The average hair length in control mice was 0.18 cm, compared to 0.40 cm in BP201-treated mice (Fig. 1C).

Hair growth promoting effect of BP201 in C3H mice

In the continuing work, C3H mice were used for further evaluating the hair growth-promoting effect of BP201. C3H mouse has been known to be an excellent animal model for human alopecia areata, since it develops hair loss that exhibits clinical, histopathological and immunohistochemical characteristics of human alopecia areata. The shaved back skins of C3H mice were treated with distilled water, BP201, MNX, or a combination of BP201 and MNX. BP201 treatment enhanced the hair growth activity of C3H mice (Fig. 2A), as indicated by 47.0- and 2.5-fold increases in the hair growth area and hair length, respectively (Fig. 2B, C). As expected, MNX treatment led to significant increases in hair growth and length (Fig. 2B, C). The combination of BP201 and MNX also enhanced hair growth activity (Fig. 2A), increasing the hair growth area and length (Fig. 2B, C) to greater extents than increases seen in groups treated with BP201 or MNX alone.

Histological analysis of hair growth promotion by BP201 in C3H mice

The numbers of hair follicles in the anagen and telogen phases of hair growth were 0 and 21.6, respectively, in the untreated control group, compared to 53.4 and 32.8, respectively, in the BP201-treated group (Fig. 3A). In BP201-treated mice, 60% of hair follicles were in the anagen phase (Fig. 3B). Similar to BP201 treatment, MNX treatment increased the total number of hair follicles (83 follicles) and the percentage of hair follicles in the anagen phase (93%) (Fig. 3A, B). The enhancement of hair growth achieved by combining the BP201
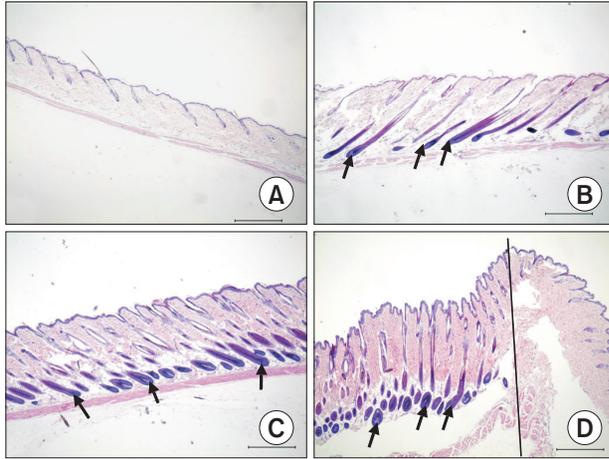
Fig. 4. Representative histological profiles of dorsal skin biopsies from the backs of C3H mice. A 60-μL sample of distilled water (A), 2% BP201 (B), 5% minoxidil (C), or 2% BP201+5% minoxidil (D) was topically applied to the shaved areas on C3H mice twice a day for 21 days. Mice were sacrificed, and dorsal skin biopsies were analyzed. Arrows show hair follicles in the anagen phase of growth. The BP201/MNX-treated area showed a higher frequency of anagen hair follicles (left panel) than the untreated area (right panel) (D). Hemotoxylin and eosin staining. Scale bars are 500 μm.

Fig. 5. Stimulating effect of BP201 on the proliferation of HFDPCs. HFDPCs were cultured for 72 h in DPC growth medium containing 0, 0.05, 0.1, or 0.2 mg/ml BP201. Viable cell numbers were determined with an MTT assay. Treatment with 0.01 μg/ml MNX led to a 1.7-fold increase in HFDPC proliferation compared to non-treated cells under the same growth conditions. Each value represents the mean ± SD of three independent experiments. *$p<0.05$; ***$p<0.001$ compared to untreated control cells.

and MNX treatments was greater than that with BP201 treatment alone, and similar to that achieved by MNX treatment alone (Fig. 3B). MNX treatment also resulted in telogen-to-anagen phase conversion of the hair growth cycle. This conversion was greater than that achieved by BP201 treatment alone (Fig. 4).

Stimulating effect of BP201 on proliferation of HFDPCs

The effect of BP201 on the proliferation of HFDPCs was examined. HFDPCs were incubated with 0.05, 0.1, or 0.2 mg/ml BP201. Treatment with BP201 stimulated the proliferation of HFDPCs in a concentration-dependent manner (Fig. 5). As expected, MNX treatment also resulted in a significant increase in the proliferation of HFDPCs.

DISCUSSION

In the present study, BP201 exhibited hair growth-promoting activities, as evidenced by the visual observation of hair regrowth on the shaved back skins of BALB/c and C3H mice, as well as the increased hair growth areas and hair lengths. The hair growth-promoting effect of BP201 was comparable to that of MNX; however, it is unclear which of the phospholipid species in BP201 is responsible for this effect.

MNX is known to slow or stop hair loss and promote hair regrowth by prolonging the anagen phase via two probable mechanisms: 1) activating Erk and Akt signaling pathways, which increase the survival of cultured DPCs; and 2) increasing the Bcl-2/Bax ratio, which protects cells against cell death (Han *et al*., 2004). In a previous study of androgenetic alopecia, retinol markedly improved the skin condition of the scalp and promoted hair growth. The combined use of MNX and retinol promoted hair growth in hair follicle organ cultures (Yoo *et al*., 2007). The combination of MNX and all-trans retinoic acid
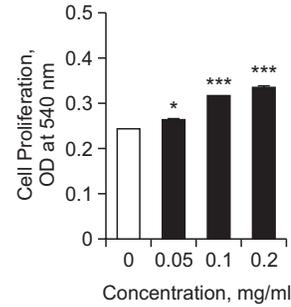
enhanced hair growth by dual functions, including the prolongation of cell survival and prevention of the apoptosis of DPCs and epithelial cells (Kwon *et al*., 2007).

BP201 was shown to possess enhanced hair growth-promoting potential when used in combination with MNX. However, it is currently uncertain how BP201, when applied in combination with MNX, would possibly diminish the side effects of MNX, including burning or irritation of eyes, itching, and redness or irritation at treated area. Since BP201 itself is a purified product of natural origin, it may be safer than MNX. A synergistic mechanism on the combinatorial application of BP201 and MNX remains to be elucidated.

Human hair follicles consist of DPCs and dermal sheath cells, which are derived from the mesenchyme. They also contain epithelial cells comprised of outer and inner root sheaths, and matrix and hair shafts, which are derived from the scalp epithelium. DPCs are one of two key components in epithelial-mesenchymal interactions that are important in the development and physiology of hair (Won *et al*., 2010). These cells play a crucial role in the induction and regulation of hair growth through paracrine and autocrine mechanisms, both in the normal hair cycle and in alopecia (Botchkarev and Kishimoto, 2003).

Androgens affect HFDPCs by generating paracrine signals that inhibit or stimulate the proliferation of follicular epithelium (Itami and Inui, 2005). Stimulation of the proliferation of HFDPCs can be one of causes for promoting hair growth. Phosphatidic acid and lysophosphatidic acid, having chemical structures analogous to phospholipids, were previously reported to promote the growth of other cell types, such as human foreskin fibroblasts, human airway smooth muscle cells and bovine aortic endothelial cells (van Corven *et al*., 1992; Cerutis *et al*., 1997; Panetti *et al*., 1997). Their effects have not been identified on the proliferation of HFDPCs. MNX was also shown to stimulate the proliferation of DPCs in human hair follicles (Han *et al*., 2004). In the present work, BP201 stimulated the proliferation of HFDPCs. However, which phospholipid species are responsible for the stimulating effect of BP201 needs to be verified in the future approaches.

In conclusion, BP201 showed hair growth-promoting potential. Treatment with BP201 enhanced the number of hair

follicles by stimulating the proliferation of HFDPCs and maintaining a high anagen-to-telogen ratio. One of its potential mechanisms would be a stimulation of a conversion of hair follicles by BP201 from telogen phase to anagen phase as a finite period of hair growth. Understanding the precise mechanisms underlying BP201 function in hair growth promotion will require further analysis.

ACKNOWLEDGEMENTS

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