Panax ginseng extract antagonizes the effect of DKK-1-induced catagen-like changes of hair follicles

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Abstract. It is well known that Panax ginseng (PG) has various pharmacological effects such as anti-aging and anti-inflammation. In a previous study, the authors identified that PG extract induced hair growth by means of a mechanism similar to that of minoxidil. In the present study, the inhibitory effect of PG extract on Dickkopf-1 (DKK-1)-induced catagen-like changes in hair follicles (HFs) was investigated in addition to the underlying mechanism of action. The effects of PG extract on cell proliferation, anti-apoptotic effect, and hair growth were observed using cultured outer root sheath (ORS) keratinocytes and human HFs with or without DKK-1 treatment. The PG extract significantly stimulated proliferation and inhibited apoptosis, respectively, in ORS keratinocytes. PG extract treatment affected the expression of apoptosis-related genes Bcl-2 and Bax. DKK-1 inhibited hair growth, and PG extract dramatically reversed the effect of DKK-1 on ex vivo human hair organ culture. PG extract antagonizes DKK-1-induced catagen-like changes, in part, through the regulation of apoptosis-related gene expression in HFs. These findings suggested that PG extract may reduce hair loss despite the presence of DKK-1, a strong catagen inducer via apoptosis.

Introduction

Hair follicles (HFs) are complicated organs composed of multiple layers of epithelia of the outer root sheath (ORS) keratinocytes, the matrix and its derivatives; the inner root sheath and hair shaft; and mesenchymal cells called the dermal papilla (DP) (1,2). The DP, which is surrounded by the dermal sheath and the hair matrix, is considered to be essential to hair induction because of secreted diffusible proteins that regulate the growth and activity of the various cells in the follicle (3,4). The ORS keratinocytes of the HF surround the hair fiber and inner root sheath. The ORS keratinocytes is distinct from other epidermal components, being continuous with the surface epidermis. The ORS keratinocytes consist of several layers of cells that can be identified by their unique ultrastructural properties (1). Hair growth and the cycling of HFs requires reciprocal interactions between the human dermal papilla cells (hDPCs) and ORS keratinocytes (5).

Apoptosis can serve a role in follicular miniaturization, but its association with androgenetic alopecia in males is controversial (6-8). Apoptosis is a complex process regulated by the Bcl-2 gene family (9). The family members act as anti- or pro-apoptotic regulators that are involved in a wide variety of cellular activities. Bcl-2, an apoptosis inhibitor, and Bax, an apoptosis promoter, show tightly regulated, hair cycle-dependent expression patterns (10). Normal HFs also express high levels of anti-apoptotic protein Bcl-2 (6,7).

Dickkopf-1 (DKK-1), which is a potent antagonist of the Wnt/β-catenin signaling pathway, is inducible by dihydrotestosterone (DHT) and promotes catagen progression and the apoptotic cell death of HFs (11). Kwack et al (12,13) demonstrated that DKK-1 is secreted from hDPCs in response to DHT and that it promotes the regression of HFs by blocking Wnt/β-catenin signaling and by inhibiting the growth of ORS keratinocytes and triggering apoptotic cell death. The reports also identified that, although DKK-1 treatment rapidly changed the anti-apoptotic protein Bcl-2, DKK-1 promoted the pro-apoptotic protein Bax in a dose-dependent manner in ORS keratinocytes.

Panax ginseng (PG) has a wide range of pharmacological effects including anti-inflammatory (14,15), antioxidant (16), anticancer (17) and anti-aging (18-22) effects as well as the promotion of hair growth (23,24). PG contains many other ingredients such as sugars, proteins and lipids besides ginsenosides. Ginsenosides are a unique component of ginseng that is found only in ginseng, while sugars and proteins are common components of other plants. Also, various studies have indicated that the pharmacological effect of ginseng is derived from ginsenosides (25,26). Recently, the authors reported that PG extract, which is a ginsenoside-enriched PG extract made using the repeated fractionalizing method,
significantly enhanced the proliferation of hDPCs, potassium channel-opening activity, and human HF growth via a mechanism similar to that of minoxidil (27). Usually, ginsenosides of commercial PG extract are 3-6%, but a ginsenoside-enriched PG extract were concentrated up to 20% using the preparation method used. The major ginsenosides detected in the ginsenoside-enriched PG extract were Rb1, Rb2, Rc, Rd, Re and Rg1. One of them, ginsenoside Re showed the highest level among the six ginsenosides and its content was approximately 6.23% (w/w) (27). In the current study, the authors investigated the inhibitory effect of ginsenoside-enriched PG extract on DKK-1-induced apoptosis in HFs in addition to the underlying mechanism of action.

**Materials and methods**

*The preparation of PG extract.* The authors conducted experiments using the same samples as PG extract, which had hair growth effect in our previous studies (27). The root of PG was obtained from Geumsan Ginseng Market (Geumsan-gun, Korea). The dried and crushed roots of PG (300 g) were extracted with 70% aqueous ethanol at 50˚C for 8 h. The extracts were filtered and concentrated under reduced pressure at 60˚C. The residue was dissolved with 100% ethanol and repeat filtration and vacuum distillation.

**Materials.** Minoxidil, MTT and dimethyl sulfoxide were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Human DKK-1 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

**Isolation and cultures of human ORS keratinocytes.** Non-balding scalp specimens were obtained from patients undergoing hair transplantation surgery (IRB:DKUH 2013-08-012-001). The medical ethical committee of the Dankook Medical College (Department of Dermatology, Cheonan, Korea) approved all of the described studies, and informed written consent was obtained from the patients. HFs were isolated and cultured by the previously described method, with minor modifications (28). Cultured ORS keratinocytes of early passage were used for the experiments and were maintained at 37˚C in a humidified atmosphere with 5% CO₂.

**MTT assay.** Cell viability was determined using an MTT assay that was performed by a slight modification of the method described by Philpott et al. (29). Briefly, ORS keratinocytes were seeded at a density of 2x10⁴ cells/well into 96-well plates and were cultured for 24 h. Prior to treatment, the cells were cultured for 24 h in a growth supplement-free medium. The cells were then treated with PG extract and DKK-1 for 24 h. The samples were assessed by measuring absorbance at 540 nm with a Synergy™ 2 Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). The cell viability rates were calculated from the optical density readings and are represented as percentages of the control value (untreated cells).

**Reverse transcription-quantitative polymerase chain reaction.** The total RNA was isolated using TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 2 µg RNA was reverse-transcribed into cDNA using SuperScript® III Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). Quantitative real-time TaqMan PCR technology (TaqMan Universal PCR Master Mix, part no. 4304437) was used (Applied Biosystems; Thermo Fisher Scientific, Inc., Santa Clara, CA, USA). The cDNA samples were analyzed to determine the expression of the following: Hs00608023_m1 (Bcl-2), Hs00180269_m1 (Bax), and Hs02758991_g1 (GAPDH). Commercially available these probes were purchased and used in Thermo Fisher Scientific, Inc.

**Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labelling (TUNEL) assay.** A TUNEL kit (In Situ Cell Death Detection kit, Fluorescein, Roche Diagnostics GmbH, Mannheim, Germany) was used according to the manufacturer’s protocol to evaluate apoptotic cells. Briefly, ORS keratinocytes at 2x10⁴ cells/200 µl were seeded into eight-chamber slides (Nunc Lab-Tek; Thermo Fisher Scientific, Inc., Roskilde, Denmark), were serum-starved for 24 h, and were then treated with PG extract and DKK-1 for 24 h. These cells were then fixed in 4% paraformaldehyde for 10 min. After being washed with PBS, the cells were incubated with 0.1% Triton X-100 in 0.1% sodium citrate for 1 h at room temperature. After washing, the cells were treated with the TUNEL reaction mixture and then were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to visualize the nuclei. Representative images were taken with a fluorescence microscope (Olympus Corp., Tokyo, Japan) at x100 magnification.

**Immunocytochemistry assay.** ORS keratinocytes at 2x10⁴ cells/200 µl were seeded into eight-chamber slides, and then treated with DKK-1 and PG extract for 24 h. These cells were then fixed in 4% paraformaldehyde for 10 min. After washing with Dulbecco's PBS, the cells were permeabized with 0.1% Triton X-100 in PBS for 10 min at room temperature and then blocked with 5% BSA in 0.05% Triton X-100 for 30 min at room temperature. The samples were incubated with Bcl-2 (1:200 dilution, sc-783; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and Bax antibody (1:200 dilution, sc-6236; Santa Cruz Biotechnology, Inc.) at 4˚C overnight. They were then washed two times with PBS and four times with distilled water followed by incubation with a Alexa Fluor™ 488 anti-rabbit (1:200 dilution, A-11034; Thermo Fisher Scientific, Inc.) and Texas Red™-X anti-rabbit (1:200 dilution, T-6391; Thermo Fisher Scientific, Inc.) secondary antibodies in 5% BSA blocking solution for 2 h at room temperature. All samples were counterstained with DAPI to visualize the nuclei. Representative images were taken with a fluorescence microscope (Olympus Corp.) at x100 magnification.

**HF organ culture and assessment of hair elongation.** Anagen HFs from human scalp skin specimens were obtained from patients undergoing hair transplantation surgery. The medical ethical committee of the Dankook University Hospital (Cheonan, Korea) approved all of the described studies. A total of six HFs/well in 24-well plates were cultured in威廉's E medium at 37˚C in a humidified atmosphere with 5% CO₂ in 500 µl basal medium supplemented with 10 µg/ml insulin, 10 ng/ml hydrocortisone, 2 mM L-glutamine, 0.1% Fungizone,
TUNEL-positive cells undergoing apoptosis significantly decreased hair shaft length and hair bulbs with minimal other histological alterations to the HFs. Either the PG extract or MNX was added at the concentration, respectively, of 20 ppm or 50 µM. The incubation medium was renewed every 2 days. The HF elongation was measured directly at 2, 5 and 7 days of culture using a light stereo microscope (Olympus Corp.).

Statistical analysis. The results are expressed as mean ± standard deviation. The data was analyzed using a Student's t-test, and the two-tailed value of P<0.05 was considered to indicate a statistically significant difference. The data was processed by SPSS software for Windows, version 22.0 (SPSS Inc., Chicago, IL, USA).

Results

PG extract stimulates proliferation and inhibits apoptosis in ORS keratinocytes. To investigate the potential role of PG extract on the proliferation and inhibition of apoptosis in ORS keratinocytes, the authors performed an MTT assay one day after treatment in the presence or absence of DKK-1 and PG extract. The PG extract and DKK-1 concentrations were determined by a previous study of the authors (data not shown). The results indicated that the PG extract enhanced the proliferation of ORS keratinocytes (Fig. 1) compared to untreated negative controls and enhanced ORS growth cultured positive controls with growth supplement medium. Treatment with DKK-1 (50 ng/ml) significantly inhibited the viability of ORS keratinocytes (Fig. 1) despite the presence of DKK-1, and it reversed the DKK-1-induced inhibition of cell viability. DKK-1 also induced the expression of pro-apoptotic factor Bax. PG extract significantly inhibited DKK-1-mediated cell reduction. Cells were treated with 50 ng/ml DKK-1 or 20 ppm PG extract or with both DKK-1 and PG extract for 1 day, and an MTT assay was assessed on day 1. ORS growth media served as a positive control for ORS keratinocyte promotion. Results were expressed as mean ± standard deviation of percentage change compared to the control. Statistically significant differences were determined by t-test (P<0.05, *P<0.01 vs. control; **P<0.05 vs. DKK-1-treated control). PG, Panax ginseng; ORS, outer root sheath; DKK-1, Dickkopf-1; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labelling; DAPI, 4',6'-diamidino-2-phenylindole.

Table I. Quantification of apoptosis in ORS keratinocytes by counting TUNEL-positive cells manually.

| Sample | Apoptosis (%) |
|--------|---------------|
| Control | 7.92±5.50 |
| DKK-1 (50 ng/ml) | 40.29±17.64* |
| PG extract (20 ppm) + DKK-1 (50 ng/ml) | 8.47±1.68** |
| Rb1 (1 µM) + DKK-1 (50 ng/ml) | 33.15±10.34* |
| Re (1 µM) + DKK-1 (50 ng/ml) | 32.30±13.01 |
| Rg1 (1 µM)+ DKK-1 (50 ng/ml) | 18.05±1.54 |

All values were expressed as mean ± standard deviation. Statistically significant differences were determined by t-test (*P<0.05 vs. control; **P<0.05 vs. DKK-1-treated control). TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labelling; DKK-1, Dickkopf-1; PG, Panax ginseng; ORS, outer root sheath.

PG extract regulates the expression of apoptosis-related genes in ORS keratinocytes. To further investigate the relevance of the anti-apoptotic effects of PG extract on DKK-1, changes in the expression of apoptosis-related genes were examined by RT-qPCR. ORS keratinocytes were treated with PG extract and/or DKK-1 for 24 h. DKK-1-induced apoptosis was accompanied by Bcl-2/Bax expression in many cells, including HF cells (12,13). In ORS keratinocytes, DKK-1 treatment significantly decreased anti-apoptotic factor Bcl-2 expression. PG extract alone increased Bcl-2 expression four times more than the untreated control, and it reversed the DKK-1-induced inhibition of Bcl-2 expression. DKK-1 also induced the expression of the pro-apoptotic factor Bax. PG extract significantly inhibited Bax expression in ORS keratinocytes (Fig. 3A) despite the presence of DKK-1. In other words, PG extract promotes ORS keratinocyte survival and increases the ratio of Bcl-2/Bax to further inhibit the cell death (Fig. 3B). Increased protein level of Bcl-2 and decreased protein of Bax were confirmed by immunocytochemistry (Fig. 3C). These results indicated that the effect of PG extract was mediated through Bcl-2/Bax expression on ORS keratinocytes.

PG extract abrogates DKK-1 inhibition of hair shaft elongation in human HF organ culture. In order to examine the effect of PG extract in the presence or absence of DKK-1 at the organ level, the authors performed an ex vivo culture of whole human scalp HFs. Minoxidil (MNX) and vehicle served as positive and negative controls, respectively. HFs treated with PG extract
grew longer than the negative control HFs at 5 days, which was similar to the growth of HFs treated with MNX. This result is consistent with the authors' previous study (27). A low dose of DKK-1 (<50 ng/ml) produced no significant impairment of hair shaft elongation compared to the vehicle (data not shown), but a dose of 50 ng/ml DKK-1 significantly inhibited hair shaft elongation. The authors observed a narrower hair bulb in HFs treated with DKK-1 at the 50 ng/ml dose, which is reminiscent of catagen-like regressive changes. They also measured the anagen/catagen ratio (Fig. 4A), and DKK-1 treatment resulted in anagen-to-catagen changes in the HF organ culture. Thus, DKK-1 treatment at the dose of 50 ng/ml was used to establish an ex vivo model of HF catagen induction. With co-incubation of the PG extract and DKK-1, the PG extract significantly abrogated DKK-1-induced growth inhibition of cultured HFs ex vivo (Fig. 4B).

**PG extract regulates the expression of hair growth-related factors in HFs.** There is evidence to suggest that several factors such as cytokine, growth factor, and apoptosis-related factor are involved in the hair growth cycle (31). In the above results, the authors already confirmed that PG extract regulates hair growth-related factors at the in vitro level. To confirm the inhibitory effect of apoptosis by PG extract at the ex vivo level, changes in the gene expression were examined by RT-qPCR using HFs treated with PG extract and/or DKK-1 for 2, 5 and 7 days. The DKK-1 treatment significantly decreased anti-apoptotic factor Bcl-2 expression at 2 days. At the same time point, the PG extract completely abolished the effect of DKK-1 on Bcl-2 expression in HFs. On the other hand, DKK-1 treatment significantly increased Bax expression in HFs, whereas the PG extract strongly inhibited this induction of Bax for 5- and 7-day HFs (Fig. 5). Of note, the PG extract affected only Bax expression and not Bcl-2 in longer ex vivo culture experiments. These results suggested that PG extract antagonizes DKK-1-induced catagen-like changes, in part, through the regulation of apoptosis-related factor expression in HFs.
The major finding of the current study is that PG extract antagonizes DKK-1-induced HF changes, resulting in hair loss. Previous studies (14-24,27) revealed that PG regulates a variety of biological effects such as anti-inflammatory, antioxidant, anticancer, and anti-aging effects, and of course, the promotion of hair growth. Recently, the authors prepared a highly concentrated ginseng extract with the repeated fractionalizing method and found that the PG extract contained 194.8 mg/g (19.48% w/w) of ginsenosides (27). Its ginsenoside content was ~3 times higher than that of commercial ginseng extracts for oral supplements in Korea and 14 times higher than that of conventional ginseng root extract (32). This newly prepared PG extract for treatment showed a significant hair growth effect with cultured hDPCs and HFs, which was comparable to the growth from minoxidil (27). Despite previous studies of PG and its effects associated with hair growth (23,24), the mechanism underlying the apoptosis response, particularly the induction of DKK-1, has not been studied with respect to PG.

DKK-1 is well known as a WNT antagonist. It induces apoptosis and inhibits the proliferation of cancer cells (33,34). DKK-1 is also inducible by dihydrotestosterone (DHT), and the level of DKK-1 is increased in the scalps of patients with male-pattern baldness compared to normal levels (11), suggesting that DKK-1 is involved in DHT-mediated balding in androgenic alopecia. It was also found that DKK-1 is highly expressed during the anagen-to-catagen transition. This implies that DKK-1 promotes the regression of HFs by blocking Wnt/β-catenin signaling and by inducing apoptosis in follicular keratinocytes (13). The authors supposed that DKK-1 might inhibit hair growth and cell proliferation via apoptosis. As shown in these results, the viability of ORS keratinocytes was decreased by DKK-1. A TUNEL assay...
demonstrated that the decreased cell viability was mediated by apoptosis.

During catagen, HFs undergo apoptosis, and there is a decline in the apoptotic protein Bcl-2 and an increase in the pro-apoptotic protein Bax (35). The ratio of these factors is important in regulating the hair cycle. In previous studies, DKK-1 treatment rapidly changed the anti-apoptotic protein Bcl-2. DKK-1 promoted the pro-apoptotic protein Bax in a dose-dependent manner (12,13). Therefore, the present study investigated the effect of PG extract in ORS keratinocytes; the extract induces apoptosis by DKK-1 in these cells. The PG extract alone significantly increased cellular proliferation. It was correlated with the mRNA level of Bcl-2 expression increase, and it also inhibited Bax gene expression (Fig. 3A and B). Furthermore, when the PG extract was co-treated with DKK-1, the effect of the DKK-1 was inhibited. This suggested that PG
extract may abolish the apoptotic signal stimulation of DKK-1 and help ORS keratinocytes to survive.

HF organ culture is now considered a useful tool for evaluating the effect of hair growth *ex vivo*. To further confirm the results shown in the above data, the authors investigated the effect of PG extract on DKK-1-induced catagen-like changes in cultured human HFs. It was shown that catagen-like morphological change was induced in DKK-1-treated HFs. Hair growth was also inhibited by DKK-1 during the incubation period. The PG extract significantly stimulated hair elongation, overcoming the inhibitory effect of DKK-1-induced hair growth, and finally, the hair was significantly more elongated than the untreated control. In summary, PG extract has the potential to protect apoptosis in HFs. These findings suggested that PG extract may enhance ORS and hDPC stimulation of HF growth despite the presence of DKK-1, a strong catagen inducer via apoptosis.

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