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Proanthocyanidins from Grape Seeds Promote Proliferation of Mouse Hair Follicle Cells *In vitro* and Convert Hair Cycle *In vivo*

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For the purpose of discovering natural products which possess hair growing activity, we examined about 1000 kinds of plant extracts concerning growth-promoting activity with respect to hair follicle cells. After an extensive search, we discovered that proanthocyanidins extracted from grape seeds promote proliferation of hair follicle cells isolated from mice by about 230% relative to controls (100%); and that proanthocyanidins possess remarkable hair-cycle-converting activity from the telogen phase to the anagen phase in C3H mice *in vivo* test systems. The profile of the active fraction of the proanthocyanidins was elucidated by thiolytic degradation and tannase hydrolysis. We found that the constitutive monomers were epicatechin and catechin; and that the degree of polymerization was 3.5. We demonstrated the possibility of using the proanthocyanidins extracted from grape seeds as agents inducing hair growth. *Key words: cell culture; condensed tannin; hair growth.*

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Many materials have been investigated since ancient times in attempts to cure male pattern baldness. However, no really effective materials were discovered until the 1980s. Minoxidil, initially prescribed in its oral form for hypertension, was found to cause hypertrichosis (1), and was approved by FDA as a medication (Rogaine®, Upjohn Co., Kalamazoo, MI, USA) for curing male pattern baldness (2). It is known that this drug stimulates the growth of hair follicle cells *in vitro* (3) and has hair cycle converting activity *in vivo* (4).

On the other hand, many plant extracts have been traditionally used for curing male pattern baldness. For instance, it has been reported that extracts of *Swertia japonica* Makino promote capillary blood flow and cause hair growth (5). Intradermal injection of capsaicin (one of the components of *Capsicum annuum* L.) into the back skin of telogen mice (C57BL/6) caused anagen induction (6). However, in most cases the efficacy was not examined and the active compounds were not identified.

We examined about 1000 kinds of plant extracts with the aim of finding hair follicle cell growth-promoting materials, and discovered proanthocyanidins extracted from grape seeds to be active compounds.

We report here on the *in vitro* growth-promoting activity with respect to hair follicle cells and the *in vivo* hair-cycle-converting activity from the telogen phase to the anagen phase possessed by proanthocyanidins extracted from grape seeds. We also propose the application of proanthocyanidins extracted from grape seeds as an active agent for curing androgenetic alopecia.

MATERIALS AND METHODS

Materials

Grape seeds (Chardonnay variety) were obtained from the Sainte Neige Wine Co. (Yamanashi, Japan). (+)-Catechin, (−)-epicatechin, (−)-epicatechin-3-*O*-gallate were purchased from the Kurita Koggyo Co. (Tokyo, Japan).

Isolation and culturing of hair follicle cells

Mouse hair follicle cells were isolated and cultured in MCDB-153 medium (7) according to the method reported by Tanigaki et al. (8) with minor modifications, which can be obtained from the authors.

Colorimetric assay for cell proliferation by MTT

The degree of cell growth was determined from an MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (9).

Preparation of topically applied agents for in vivo evaluation

Fourteen grams of ethyl alcohol, 0.6 g of proanthocyanidins purified from the grape seeds, 2 g of 1,3-butylene glycol, 0.1 g of isostearyl *N*-acetylglutamine (Kyowa Hakko Kogyo Co., Japan), 0.05 g of polyoxyethylene (25) glyceryl monopyroglutamate monoisostearate (Nihon Emulsion Co., Japan), and 3.25 g of pure water were mixed, whereby the solids were dissolved to prepare a sample solution for the *in vivo* mice test.

Vehicle without proanthocyanidins was used as the control. Minoxidil and other drug-containing agents were prepared in the same way as the proanthocyanidin-containing agent.

Test for hair-cycle-converting activity in mice

With reference to the method of Hattori & Ogawa (10), the hair-cycle-converting activity was measured. In this test, 8-week-old male C3H/HeS1c mice whose hair cycle was in the telogen stage were used (11).

Purification of proanthocyanidins from grape seeds

Dry grape seeds (Chardonnay variety) was extracted with 75(v/v)% acetone, further purified using a column (9 cm × 28 cm size with a volume of 1780 ml) filled with Diaion HP-20 resin (Mitsubishi Kasei Co., Japan) followed by preparative high-performance liquid chromatography (HPLC) using an ODS column.

Preparation of procyanidin B-2 [epicatechin-(4β→8)-epicatechin]

Apple juice was applied to an HP-20 column (15(v/v)% methanol wash and 40(v/v)% methanol eluate), next applied to an LH-20 column (Pharmacia Biotech Co., Sweden, 50(v/v)% methanol wash and 75(v/v)% methanol eluate), followed by preparative HPLC (ODS column, mobile phase was 15(v/v)% methanol).

Preparation of procyanidin B-3 [catechin-(4α→8)-catechin]

Barley husk was extracted with 70(w/w)% acetone; this extract was evaporated, and the following column purification proceeded by the same procedure as that of procyanidin B-2.

Determination of the structure of proanthocyanidins

The profile of the extracted and purified proanthocyanidins was characterized by the composition of the flavan-3-ol units (Fig. 1), the degree of polymerization and the degree of galloylation. The

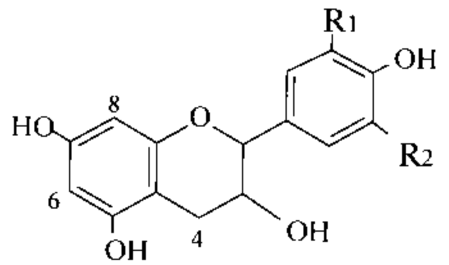

Fig. 1. Composition of constitutive units of proanthocyanidins; R1 and R2 represent a hydrogen atom or a hydroxyl group. Proanthocyanidins are oligomers and polymers comprising C-4 to C-8 or C-4 to C-6 linked flavan-3-ol units.

proanthocyanidins and the tannase-treated proanthocyanidins (12) were degraded by toluene-α-thiol (13).

Hydrolysis with tannase

A dry sample (16.3 mg) was dissolved into 500 μl of 0.1 M sodium acetate (pH 5) containing tannase (0.5 international units (IU), Kikkoman Corp., Japan) and incubated at 30°C for 16 h (12). The products were analyzed by HPLC.

Thiolytic degradation

A dry sample (5.6 mg) was dissolved in ethanol (1.35 ml) containing 11% toluene-α-thiol and 15% acetic acid and refluxed at 70°C for 24 h under nitrogen atmosphere (13). The products were analyzed by HPLC.

RESULTS

Search for hair follicle cell growth-promoting materials from plant extracts

About 1000 kinds of extracts were prepared by using three different solvents (chloroform, methanol and boiling water), from the roots, leaves, fruits and seeds of 132 plant species: the list can be obtained from the authors. We examined the proliferative activity of the extracts with respect to hair follicle cells.

The methanol extract of grape seeds showed significant proliferative activity. After repeated fractionation and measurement of proliferative activity with respect to hair follicle cells, proanthocyanidins were identified as the active compounds.

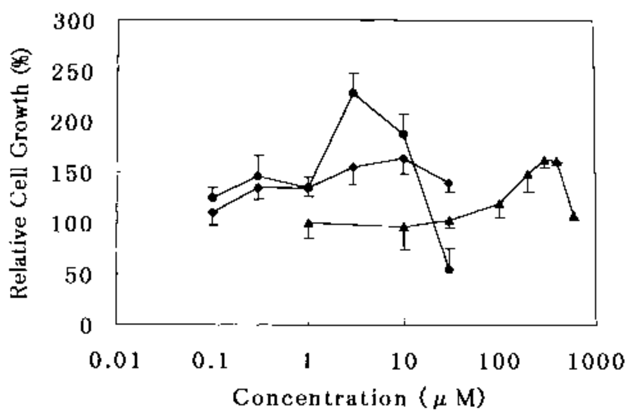

Fig. 2. Growth-promoting activities for hair follicle cells of proanthocyanidins purified from grape seeds (●), (−)-epicatechin (◆) and minoxidil (▲) by MTT assay. Cell growth was indicated by percentage relative to controls (=100%) in a 5-day culture.

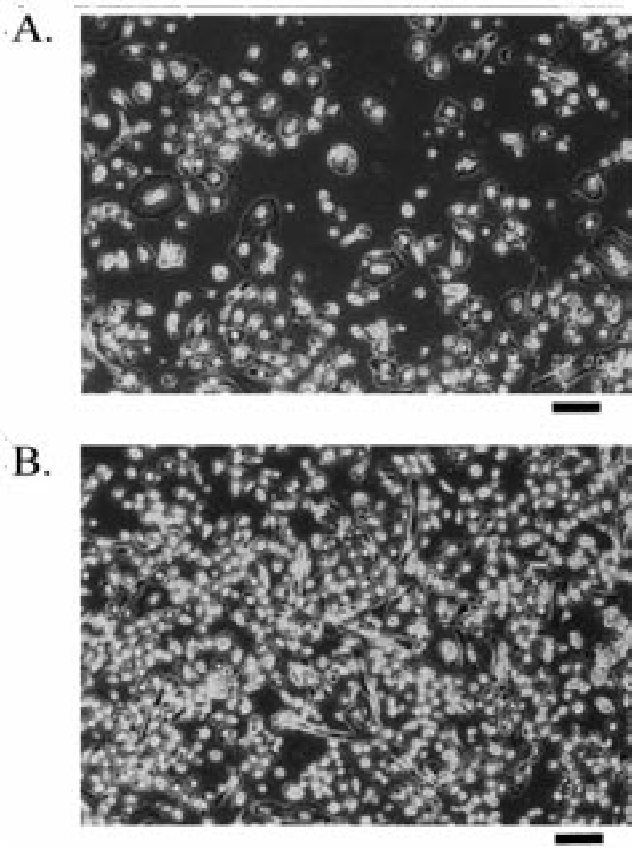

Fig. 3. Micrographs of hair follicle cells from mice cultured in MCDB-153 medium for 5 days; control (A), 3 μM proanthocyanidins purified from grape seeds (B). Magnification ×25, bar = 100 μm.

Hair follicle cell culture

Fig. 2 shows the relative growth in MTT assay with the proliferative capacity of the controls normalized at 100. Proanthocyanidins extracted from grape seeds promote the proliferation of mouse hair follicle cells *in vitro* at about 230% relative to controls at a concentration of 3 μM in a 5-day culture. Minoxidil was less effective in this cell culture system, with a proliferative activity of about 160% at a concentration of 400 μM.

Epicatechin, a proanthocyanidin monomer, was less effective than proanthocyanidins in oligomeric form. The proliferative activity of epicatechin was about 160% at the optimum concentration of 10 μM.

Fig. 3 shows micrographs of hair follicle cells cultured in MCDB-153 medium for 5 days. After culturing in the proanthocyanidins-containing medium, the hair follicle cells appear to have adopted a rounded shape.

Hair cycle conversion assay using C3H mice

C3H mouse dorsal hair is known to have a time-synchronized hair growth cycle. From about 2.5 to 3.5 weeks old and 5 to 14 weeks old; the dorsal hairs are in the telogen phase. From 0 to 2.0 weeks old and 4.0 to 4.5 weeks old; the dorsal hairs are in the anagen phase. The test compound was topically applied from the 8th to the 10th week (19-day application) during the second telogen phase, and the hair-covered area at the 10th week was evaluated.

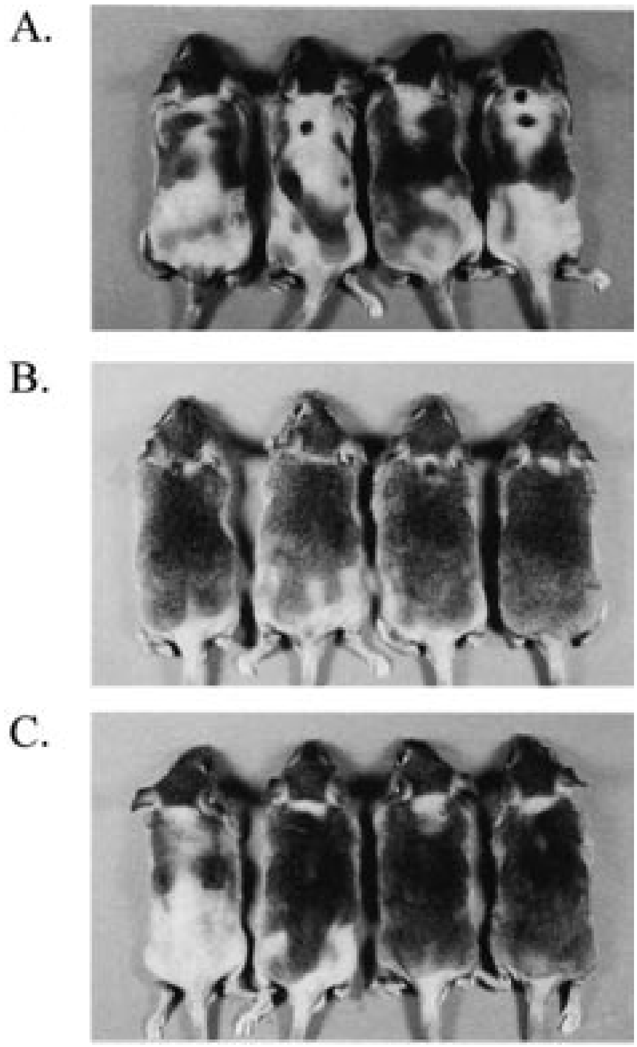

Fig. 4. The photographs of mice after topical application of vehicle as a control (A), 1% minoxidil (B), and 3% proanthocyanidins purified from grape seeds (C). These agents were applied to 8-week old C3H mice (♂), 200 μl/day/mouse for 19 days.

In this assay system, minoxidil gave a positive response. After a 19-day application of 1% minoxidil-containing solution, about 90–100% of the shaven area showed emergence of hair. The control group to which vehicle was applied, on the other hand, showed little hair growth: only about 30–40% of the shaven area was covered with hair on day 19. The group to which 3% proanthocyanidins purified from grape seeds had been applied showed an extensive growth area of about 80–90% of the shaven area on day 19 (Fig. 4). On the other hand, epicatechin showed no hair-cycle-converting activity *in vivo*. No inflammation or other side effects were observed in any of the groups.

Structure of proanthocyanidins purified from grape seeds

To determine the structure of proanthocyanidins purified from grape seeds, we used a combination of thiolytic degradation (13) and hydrolysis by tannase (12), followed by reverse-phase HPLC analysis. The thiolytic degradation method can be used to distinguish between extension units and terminal units, because extension units are released in benzylthioether form, while terminal units are released as flavan-3-ols.

Table I. *Characteristics of the active fraction of proanthocyanidins obtained from grape seeds. Composition of flavan-3-ol units*

	Catechin	Epicatechin	Epicatechin gallate
Terminal units	1	2.8	1.3
Extension units	2.3	8.1	3.4

The profile of the active fraction of proanthocyanidins extracted from grape seeds is shown in Table I. Catechin, epicatechin and epicatechin-3-*O*-gallate were the only monomers released by thiolytic degradation. As for the compositions of the flavan-3-ol units, the molar ratio of epicatechin (including gallate conjunct) to catechin was 4.7, revealing epicatechin to be the major component of its constitutive units. The average degree of polymerization was calculated to be 3.5. The extent of galloylation was calculated to be 25% at the molar ratio per constitutive flavan-3-ol unit.

DISCUSSION

Proanthocyanidins

Proanthocyanidins (14), a species of condensed tannin, are phenolic oligomers and polymers comprising C-4 to C-8 (or C-4 to C-6) linked flavan-3-ol units, such as catechin and epicatechin (Fig. 1). Proanthocyanidins exist commonly in plants, for example in grape seeds (15), apple juice (16), pine sap (17) and palms (18). Proanthocyanidins have many phenolic hydroxyl groups in their molecules and are known to have antioxidant properties (19).

Proanthocyanidins are used commercially as food additives. It has been reported that proanthocyanidins possess radical scavenging activity (20), antimutagenic activity (21), anti-tumor-promoting effects (22), antifungal activity (23), anti-ulcer activity (24), capillary protective action (25), and anti-hypertensive effects (26). We have found a new facet of proanthocyanidins: hair-growing activity.

Our analysis of the active fraction of proanthocyanidins extracted from grape seeds showed the profile of the proanthocyanidins to be a relatively smaller oligomeric molecule than that of proanthocyanidins extracted from grape seeds reported by Prieur et al. (15). We are now investigating which molecules in proanthocyanidins possess higher proliferative activity and hair-cycle-converting activity.

Presumed mechanism of action of the proanthocyanidins

In recent years, the "bulge hypothesis", which was first reported by Cotsarelis et al. (27), has been generally accepted. According to this hypothesis, the stem cell exists in the bulge area of the outer root sheath. Kobayashi et al. reported the rates of colony formation of sectioned hair follicles, and high colony formation rate was observed over the bulge region (28, 29). It is assumed that in the hair follicle cell culture system, the outer root sheath cells account for the major portion of the growing cell population from the culture conditions: MCDB-

153 developed for the culture of epidermal keratinocytes was used (30). The morphology of hair follicle cells cultured in proanthocyanidins-containing medium took on the rounded appearance characteristic of undifferentiated juvenile cells (Fig. 3). This suggests that the mechanism of action of proanthocyanidins may involve the prevention of cell differentiation and the retention of the growing phase. It is assumed that the growth-promotive effects of proanthocyanidins on the outer root sheath cells switch the bulb region to the growing phase by some mechanism, causing the follicular hair cycle to convert from the telogen phase to the anagen phase.

It is interesting that epicatechin, a monomer of proanthocyanidins, does not possess hair-growing activity, and that hair growth appears to be dependent on the oligomeric structures of proanthocyanidins. As for the physiological effects of minoxidil, Ohtsuyama & Morohashi report that a reduction of the internal calcium concentration of hair follicle cells (31) was observed in reaction to dosing with minoxidil. It is possible that the effects caused by proanthocyanidins are caused by the modulation of certain signal transduction cascades. Minoxidil showed growth-promoting effect on hair follicle cells; however, the potential was relatively weaker than that demonstrated by proanthocyanidins (Fig. 2). Routes other than direct action on hair follicle cells have been suggested as the mechanisms of action of minoxidil for hair growth. However, the chief mechanism of action of proanthocyanidins is thought to be direct action on hair follicle cells.

Potential for the use of proanthocyanidins as agents inducing hair growth

We have demonstrated that proanthocyanidins extracted from grape seeds possess both hair follicle cell growth-promoting activity and hair-cycle-converting activity *in vivo* similar to minoxidil. The fact that proanthocyanidins showed the same positivity in C3H mouse *in vivo* test systems as minoxidil led to consideration of the potential for application of proanthocyanidins to androgenetic alopecia.

Proanthocyanidins are now used as skin conditioners and have been shown to have no side effects such as inflammation or stimulation (32). We are now investigating the possibility of the use of proanthocyanidins as agents for curing androgenetic alopecia.

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