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Procyanidin B-3, isolated from barley and identified as a hair-growth stimulant, has the potential to counteract inhibitory regulation by TGF-β1

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Abstract: With the aim of identifying natural products, which possess hair-growing activity, we examined more than 1000 plant extracts with respect to their growth-promoting effects on hair epithelial cells. We discovered intensive growth-promoting activity, about 140% relative to controls, in barley extract. Our strategy for identifying active compounds in barley extract involved subjecting it to column chromatography using HP-20 resin columns, an LH-20 resin column, and preparative high-performance liquid chromatography (HPLC) using an ODS column. The 60% (v/v) aqueous methanol eluted fraction from the HP-20 column and the 75% (v/v) aqueous methanol eluted fraction from the subsequent LH-20 column showed high hair-growing activity *in vivo*. We isolated two major substances from the LH-20 active fraction using preparative HPLC. By means of mass spectrometry, ^{1}H-NMR, and ^{13}C-NMR analyses, one substance was revealed to be procyanidin B-3 and the other substance was identified as (+)-catechin. Purified procyanidin B-3 showed high hair-growing activity in the form of *in vitro* hair epithelial cell growth-promoting activity and *in vivo* anagen-inducing activity; however (+)-catechin showed no hair-growing activity. For the purpose of examining the hair-growing mechanisms of procyanidin B-3, we examined its relationship to the TGF-β signal pathway, which is known to be a regulator of catagen induction. Addition of TGF-β1 to hair epithelial cell cultures dose-dependently decreased the cell growth, and addition of procyanidin B-3 to the culture neutralized the growth-inhibiting effect of TGF-β1. From these results, it is concluded that procyanidin B-3 can directly promote hair epithelial cell growth *in vitro*, has the potential to counteract the growth-inhibiting effect caused by TGF-β1 *in vitro*, and has potential to stimulate anagen induction *in vivo*.

Ayako Kamimura and Tomoya Takahashi

Tsukuba Research Laboratories, Kyowa Hakko Kogyo Co., Tsukuba, Ibaraki, Japan

Key words: hair – mouse – proanthocyanidins – skin – TGF-β

Ayako Kamimura, Tsukuba Research Laboratories, Kyowa Hakko Kogyo Co., 2, Miyukigaoka, Tsukuba, Ibaraki 305-0841, Japan
Tel.: +81 298 56 4282
Fax: +81 298 56 4288
e-mail: ayako.kamimura@kyowa.co.jp

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Introduction

The hair follicle is composed of dermal papilla cells, which are classified into mesenchymal cells, and epithelial cells, such as the inner root sheath cells, outer root sheath cells, and hair matrix cells (1). Interactions between mesenchymal cells and epithelial cells are thought to be important both in hair morphogenesis in the embryo (2) and in the hair cycle progression in adult skin (3,4). These mesenchymal-epithelial interactions are assumed to stimulate the germinative cells in hair follicles to proliferate and induce the anagen phase of the hair cycle (5); and these interactions are also assumed to activate stem cells by direct or indirect mechanisms and promote down-growth of outer root sheath cells, resulting in the formation of mature hair follicles (6).

Several internal factors are known to regulate hair growth (7): for example, IGF-1 (8) and HGF (9, 10) are known to positively regulate hair growth; TGF-β (11), FGF-5 (12, 13), TNF-α (14), IL-1α

(14–16), and IL-1β (14, 17) are known to negatively regulate hair growth. On the other hand, external factors, such as plant-derived compounds, metabolites of microorganisms, and synthetic drugs, are also known to promote hair growth. Cepharantin (18), cyclosporin A (19), and minoxidil (20) are known to promote hair epithelial cell growth or hair shaft growth in *in vitro* tissue culture or organ culture. Cutaneously injected capsaicin (21), externally applied cyclosporin A (22), and externally applied minoxidil (23) are also known to induce the anagen phase of the hair cycle in animal models. Cyclosporin A (24), diazoxide (25), and minoxidil (26) are known to cause hirsutism in humans. Minoxidil (Rogaine®, Upjohn Company, Kalamazoo, MI, USA) (27, 28) is practically used to treat male pattern baldness.

Many plant-derived materials have been traditionally used and applied in hair-growing agents. However, most plant-derived substances are used in a supportive rather than curative fashion to keep the skin in good health, with the intent of promoting the circulation or affording anti-inflammatory or antimicrobial effects. Little is known about compounds that directly stimulate hair growth, and no plant-derived substances have up to now been identified as active ingredients that show clear and intensive hair-growing effects.

For the purpose of discovering natural products, which directly activate hair follicles, we examined more than 1000 kinds of plant extracts for growth-promoting effects on hair epithelial cells. We assembled a collection of more than 130 pharmaceutically usable plants and edible plants, and divided each plant into several sections such as leaves, roots, seeds, and fruit. We then applied solvent extraction to the samples: first by chloroform, then by methanol, and finally by hot water. In all, we prepared more than 1000 plant extracts. We then examined the growth-promoting activity of these extracts on hair epithelial cells, which we had isolated from C3H mouse dorsal skin. We found activity in methanol extract from seeds of barley, about 140% growth-promoting activity relative to the controls. To identify the active ingredients in barley extract, we subjected it to column purification. After repeated fractionations and measurements of hair-growing activity using *in vivo* C3H mice, we finally isolated procyanidin B-3 as the active ingredient. Recently, we have found hair epithelial cell growth-promoting activity and ability to induce the anagen phase *in vivo* in proanthocyanidins purified from grape seeds, which have characteristically high molecular weights and possess gallate in their molecules (29). The barley-derived active compound described in this paper is coincidentally the same type of compound, a procyanidin oligomer.

In this report, we describe the purification process to reach procyanidin B-3, the active ingredient in barley, and the results of hair-growing activity tests on each fraction after each purification step. We focus on the comparison of the activities of the purified prodelphinidin-rich fraction, the procyanidin polymer-rich fraction, and the procyanidin oligomer-rich fraction. We also discuss the mechanisms by which procyanidin B-3 exerts hair-growing activity.

Materials and methods

Materials

Authentic standards of (+)-catechin and procyanidin B-3 [(+)-catechin-(4αr)8)-(+)-catechin] were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TGF-β1 (human recombinant) was purchased from Sigma.

Preparation of barley extract and purification process of active fraction from barley extract by means of column chromatography

Thirty kilograms of seed husks of barley (*Hordeum vulgare* L. var. *distichon* Alefeld) were extracted with 90 kg of 75% (v/v) aqueous acetone at room temperature for 4 days. The extract was filtered through a 212-μm stainless-steel mesh and through a 15-μm-nylon mesh, and then evaporated to produce 2100 g of a dry solid. This was then dissolved in demineralized water, and applied to an HP-20 column (15 cmϕ × 50 cm; volume: 8.8 litres) that had been equilibrated with demineralized water. After washing the column with 18 litres of demineralized water and 18 litres of 20% (v/v) aqueous methanol, 18 litres of 100% methanol was applied to the column. The eluate was evaporated to produce 173 g of a dry solid (Fraction A). Fraction A was then dissolved in demineralized water, and applied to an HP-20 column (12 cmϕ × 70 cm; volume: 8 litres) that had been equilibrated with demineralized water. After washing the column with 16 litres of demineralized water and 16 litres of 20% (v/v) aqueous methanol, 16 litres of 40% (v/v) aqueous methanol was applied to the column and the fraction was obtained and evaporated to produce 38.5 g of a dry solid (Fraction B). Then, 16 litres of 60% (v/v) aqueous methanol was applied to the column and the fraction obtained was evaporated to produce 22.1 g of a dry solid (Fraction C). Next, 16 litres of 80% (v/v) aqueous methanol was applied to the column and the fraction obtained was evaporated to produce 33.2 g of a dry solid (Fraction D). Fraction C (20.0 g) was then dissolved in 400 ml of 25% (v/v) aqueous methanol and applied to a column (8 cmϕ × 40 cm; volume: 2 litres) packed with Sephadex LH-20 (Pharmacia Biotech Co., Uppsala, Sweden) and equilibrated with 25% (v/v) aqueous methanol. After washing the column with 4 litres of 25% (v/v) aqueous methanol, 4 litres of 50% (v/v) aqueous methanol was applied to the column. The fraction obtained was evaporated to produce 2.6 g of a dry solid (Fraction E). Then, 4 litres of 75% (v/v) aqueous methanol was applied to the column and the fraction obtained was evaporated to produce 1.7 g of a dry solid (Fraction F). Next, 4 litres of 100% methanol was applied to the column and the fraction obtained was evaporated to produce 3.1 g of a dry solid (Fraction G). Fraction F (1.2 g) was then dissolved in demineralized water and subjected to preparative high-performance liquid chromatography (HPLC) (ODS column, 15 μm particle size, 3 cmϕ × 30 cm, Nihon Waters, Ltd, Tokyo, Japan), which was equilibrated with demineralized

water. The eluting conditions were as follows: flow-rate, 20 ml/min; room temperature; mobile phase A = 0.0001% (v/v) acetic acid, mobile phase B = methanol; isocratic elution with A = 88%, B = 12%; monitored by a UV detector at a wavelength of 280 nm. Finally, 159 mg of procyanidin B-3 and 108 mg of (+)-catechin were obtained. The product was identified by mass spectrometry, ^{1}H-NMR, and ^{13}C-NMR (30–32).

Colorimetric assay of proanthocyanidins

The proanthocyanidin content in each fractionation sample was measured by vanillin assay according to the method described by Price et al. (33). Briefly, a 0.1 ml sample was added to 2.0 ml of 0.5% (w/v) vanillin and 4% (v/v) concentrated HCl in methanol; and the reaction was carried out in a 30°C water bath for 20 min, after which absorbance at 500 nm was measured. For the standard, procyanidin B-2 purified from apples (34) was used.

Isolation and culturing of hair epithelial cells

Murine hair epithelial cells were isolated from C3H/HeNCrj mice (Charles River Japan, Inc., Kanagawa, Japan) (35). The dorsal skin was peeled from 4-day-old C3H/HeNCrj mice, cut into about 5 mm widths, then dipped into Eagle's minimum essential medium (MEM) containing 750 IU/ml dispase (from *Bacillus polymyxa*, Godo Shusei Co., Tokyo, Japan), 60 mg/l kanamycin, and 10% fetal calf serum (FCS) at 4°C for 20 h. The epidermis was peeled off, and the remaining dermis layer was dispersed in Dulbecco's modified Eagle medium (DMEM) containing 0.25% collagenase (from *Streptomyces parvulus*, Nitta Gelatin Co., Osaka, Japan), 50 000 U/l penicillin, 50 mg/l streptomycin, 0.5% bovine serum albumin (BSA) and 20% FCS at 37°C for 1 h, stirring occasionally. This dermis suspension was filtered through a 212-μm-nylon mesh, and the filtrate was centrifuged at 1400 r.p.m. (400 g) for 7 min. The pellet was resuspended in Dulbecco's phosphate-buffered calcium- and magnesium-free saline containing 50 000 U/l penicillin and 50 mg/l streptomycin (PBS-PS). The suspension was left to stand for 15 min, allowing the hair follicle tissue to precipitate, after which the supernatant was removed using an aspirator. The hair follicle tissue was resuspended in PBS-PS and then precipitated. This precipitation process was repeated three times. Finally, the hair follicle tissue was incubated in 0.05% EDTA-0.25% trypsin in Hanks' balanced calcium- and magnesium-free salt solution (HBSS) (Life Technologies, Inc., MD, USA) at 37°C for 5 min. The hair follicle cells were suspended in DMEM supplemented with 50 000 U/l penicillin, 50 mg/l streptomycin, and 10% FCS at a density of 3×10^5 cells/ml after filtration via a 212-μm-nylon mesh. This hair follicle cell suspension was pipetted into a 24-well Type I collagen-coated plate (2 cm^2/well, Iwaki Glass Co., Chiba, Japan) at a rate of 1 ml/well and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 24 h. After a 24-h incubation, the medium was exchanged with MCDB 153 (Sigma) containing 5 mg/l bovine insulin, 5 μg/l mouse EGF, 40 mg/l bovine pituitary extract, 10 mg/l human transferrin, 0.4 mg/l hydrocortisone, 0.63 μg/l progesterone, 14 mg/l *O*-phosphorylethanolamine, 6.1 mg/l ethanolamine, 50 000 U/l penicillin, and 50 mg/l streptomycin. It was then further incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 5 days. During incubation, the medium was removed and replaced with fresh medium every other day.

Colorimetric assay for cell proliferation by MTT

The degree of cell growth was determined by means of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (36). To summarize, MTT reagent was dissolved in Dulbecco's phosphate-buffered calcium- and magnesium-free saline (PBS) at a concentration of 5 mg/ml, and filtered through a 0.45-μm membrane filter (cellulose acetate, DISMIC-13cp, Advantec, Tokyo, Japan), and added 10% (v/v) to the culture medium. The culture plate was further incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 4 h. After removing the medium, the formed dye was extracted with acidic isopropanol containing 0.04 N HCl (adding 1.0 ml per 2 cm^2 well), and the absorbance was measured at 570 nm relative to 640 nm.

Preparation of topically applied agents for in vivo evaluation

Test samples were dissolved in basal solvent comprising 70% (w/w) ethyl alcohol, 10% (w/w) 1,3-butylene glycol, 0.5% (w/w) *N*-acetylglutamine isostearyl ester (Kyowa Hakko Kogyo Co., Tokyo, Japan), 0.25% (w/w) polyoxyethylene (25) glyceryl monopyroglutamate monoisostearate (Nihon Emulsion Co., Tokyo, Japan), and pure water making up to 100%; and subjected to *in vivo* mouse testing. Basal solvent (= vehicle) was used as the control.

Test for hair-growing activity by induction of the anagen phase using the C3H mouse model

The degree of hair-growing activity by induction of the anagen phase was measured using C3H mice with reference to the method introduced by Hattori and Ogawa (37). In this test, 8-week-old male C3H/HeSlc mice (Japan SLC, Inc., Shizuoka, Japan) whose hair cycle was in the telogen phase were used. The mice were placed into several groups, each containing four or five mice. The hair on the back of each mouse was carefully shaven with an electric shaver so as not to injure or stimulate the skin. Two hundred microliters daily of test sample was applied to the shaven area. On the 19th day of the test, the mouse back skin was observed and photographed; then the skin was peeled from the back of each mouse and photographed.

Results

Crude extract of barley husks promotes hair epithelial cell growth

Barley husks were immersed in 75% (v/v) aqueous acetone and the filtrate was evaporated and obtained as a crude extract (hereafter referred to as barley extract) (Fig. 1). Addition of 100 μg/ml barley extract to the culture medium promoted murine hair epithelial cell growth at about 140% relative to the controls (= 100%) in a 5-day culture (data not shown). We confirmed that barley extract contains substances that promote hair epithelial cell growth.

Partially purified active fraction of barley extract promotes hair epithelial cell growth and dose-dependently stimulates anagen induction in the hair cycle progression in tests using a C3H mouse model

Seventy-five percent (v/v) aqueous acetone extract of barley husks was applied to a HP-20 column.

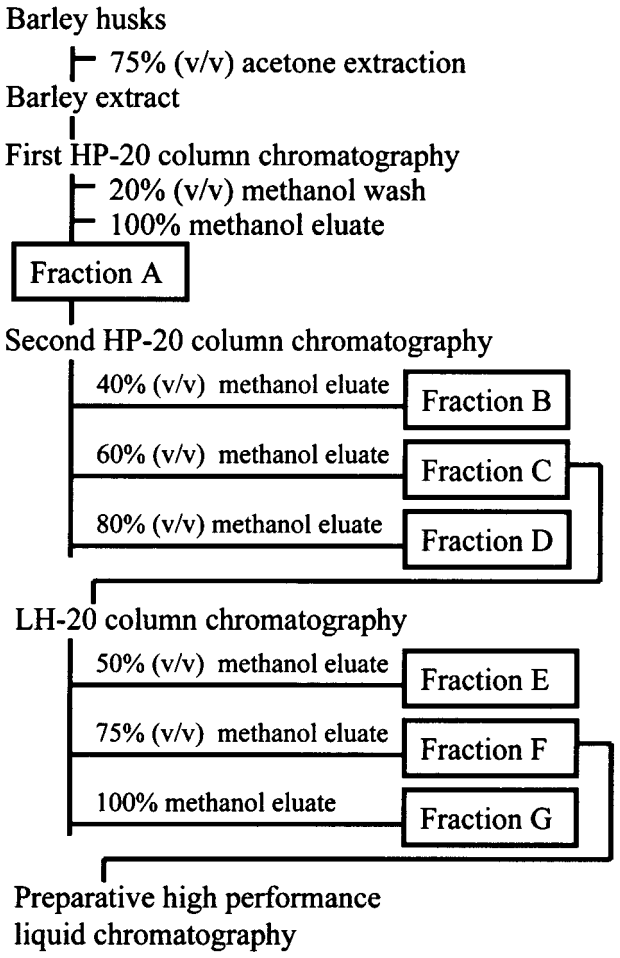

Figure 1. Purification process of active fractions from barley husks. Thirty kilograms of barley husks was extracted using 90 kg of 75% (v/v) aqueous acetone. The extract was then evaporated and dissolved in demineralized water and applied to the first HP-20 column; after washing the column with 20% (v/v) aqueous methanol, it was eluted with 100% methanol to obtain Fraction A. Fraction A was then applied to the second HP-20 column and eluted with aqueous methanol to obtain Fraction B [40% (v/v) aqueous methanol elution], C [60% (v/v) aqueous methanol elution], and D [80% (v/v) aqueous methanol elution]. Fraction C was then applied to an LH-20 column and eluted to obtain Fraction E [50% (v/v) aqueous methanol elution], F [75% (v/v) aqueous methanol elution], and G (100% methanol elution). Fraction F was then subjected to preparative high-performance liquid chromatography (HPLC) (ODS column) and procyanidin B-3 and (+)-catechin obtained.

Fraction A was obtained using the scheme shown in Fig. 1. We examined the growth-promoting activity on murine hair epithelial cells of Fraction A. Addition of Fraction A to the culture medium promoted hair epithelial cell growth at a maximum rate of 140% relative to controls (= 100%). Optimum concentration was 3 μg/ml (Fig. 2). A micrograph of mouse hair epithelial cells cultured in Fraction A-containing medium is shown in Fig. 3. Next, we examined Fraction A's hair-growing activity effected by induction of the anagen phase of the hair cycle. After a 19-day application, the hair-growing area of the vehicle-applied group was 29.5% ± 12.9% (average ± SD); on the other hand, the groups to which Fraction A had been applied showed a dose-dependent increase in the hair-growing area: the 1% (w/w) Fraction A-applied group was 49.7% ± 19.6%; the 5% (w/w) Fraction A-applied group was 76.6% ± 26.9%; and the 8% (w/w) Fraction A-applied group was 89.5% ± 8.0% (Figs 4 and 5). We confirmed that Fraction A dose-dependently stimulated anagen induction in tests using the C3H mouse model.

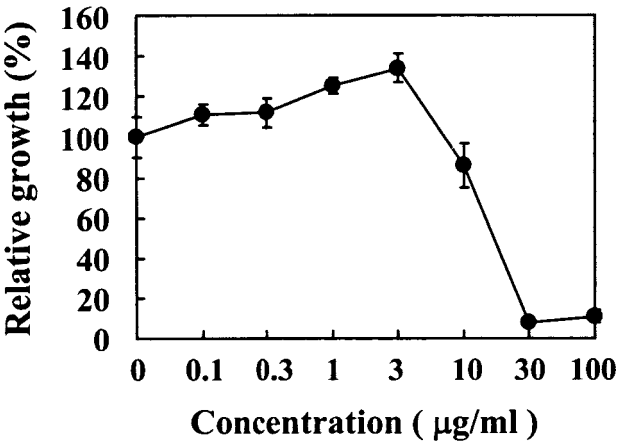

Figure 2. Partially purified active fraction of barley extract intensively promotes hair epithelial cell growth. Growth-promoting activities on hair epithelial cells relative to controls (= 100%) are shown. Fraction A obtained from barley extract using the scheme shown in Figure 1 was added to the culture during the last 5 days. Medium without Fraction A was used as the control. Results are represented as mean ± SD ($n = 6$) carried out with primary cultures prepared from 50 neonatal mice.

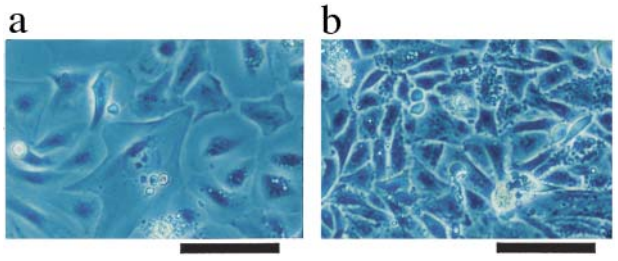

Figure 3. Micrographs of hair epithelial cells from mice cultured in MCDB 153 medium for 5 days. (a) Control and (b) 3 μg/ml Fraction A obtained from barley extract. Bar = 100 μm.

Further purification of the active fraction of barley extract using columns packed with HP-20 resin and LH-20 resin, followed by preparative HPLC using an ODS column

The hair-growing areas of the dorsal skin after 19-day application of fractions obtained by second

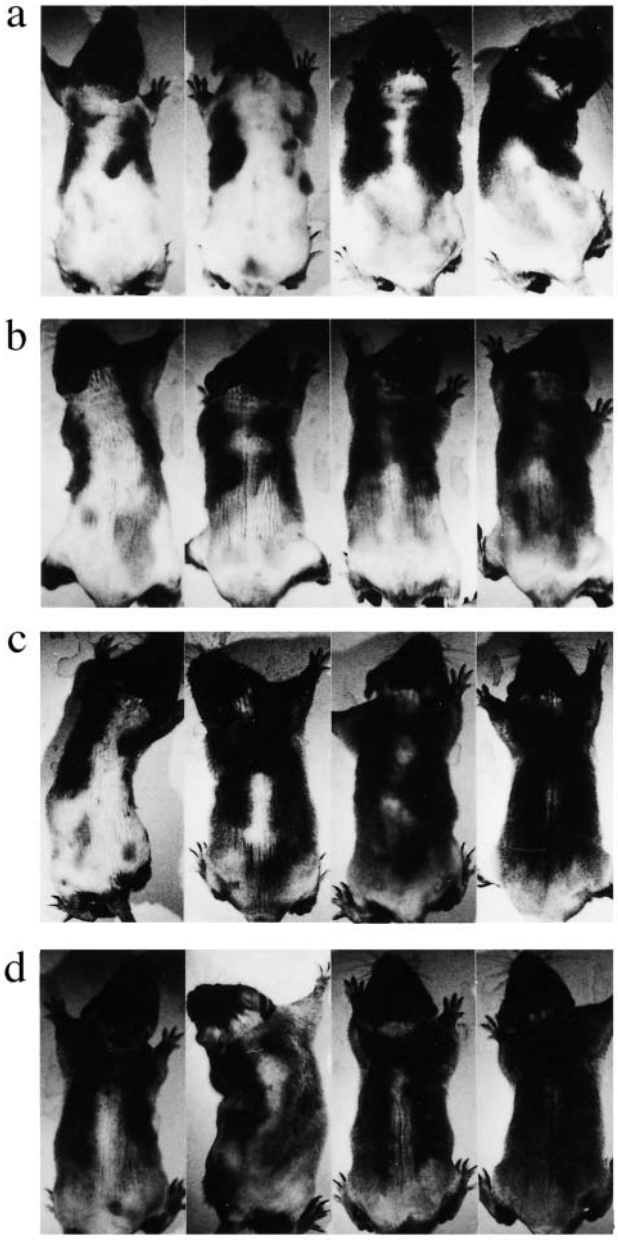

Figure 4. Partially purified active fraction (Fraction A) from barley extract using an HP-20 column was able to induce the anagen phase efficiently in the hair cycle progression in the murine model. Photographs were taken after topical application of test agents for 19 days. Test agents were applied to 8-week-old male C3H telogen mice at 200 µl/day/mouse. (a) Vehicle, (b) 1% (v/v) Fraction A from barley extract, (c) 5% (v/v) Fraction A from barley extract and (d) 8% (v/v) Fraction A from barley extract.

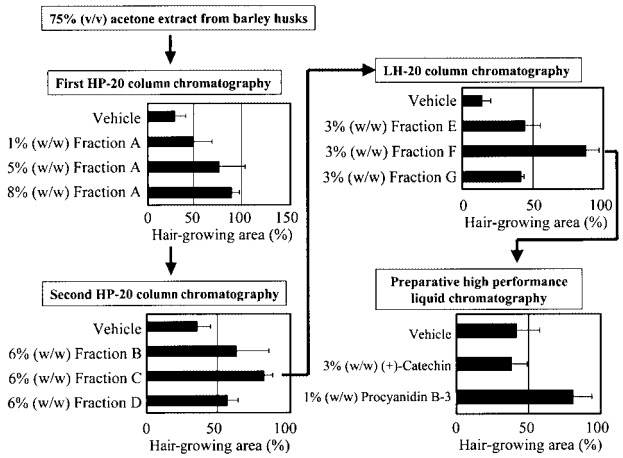

Figure 5. The hair-growing activity of each fraction of each purification step is shown. The results of animal experiments shown in Figs 4, 6, 7, and 10 are demonstrated in bar graphs.

HP-20 column chromatography are as follows: the vehicle-applied group was 36.7% ± 8.6% (average ± SD); the 6% (w/w) Fraction B-applied group was 63.2% ± 22.4%; the 6% (w/w) Fraction C-applied group was 82.1% ± 6.0%; and the 6% (w/w) Fraction D-applied group was 56.9% ± 7.6% (Figs 5 and 6). In second HP-20 column chromatography, Fraction C, which was eluted with 60% (v/v) aqueous methanol through the HP-20 column to which Fraction A had been applied, showed marked hair-growing activity by induction of the anagen phase of the hair cycle.

The hair-growing areas of the dorsal skin after 19-day application of fractions obtained by LH-20 column chromatography are as follows: the vehicle-applied group was 14.2% ± 6.5% (average ± SD); the 3% (w/w) Fraction E-applied group was 44.7% ± 10.9%; the 3% (w/w) Fraction F-applied group was 87.7% ± 9.3%; and the 3% (w/w) Fraction G-applied group was 41.5% ± 2.2% (Figs 5 and 7). After LH-20 column chromatography, Fraction F, which was eluted with 75% (v/v) aqueous methanol through the LH-20 column to which Fraction C had been applied, showed marked hair-growing activity by induction of the anagen phase of the hair cycle.

HPLC analysis of Fraction F using an ODS column monitored by a UV detector set at 280 nm showed that Fraction F contains two major substances (Fig. 8). We next subjected Fraction F to preparative HPLC and obtained these two substances. ^{1}H-NMR, ^{13}C-NMR, and mass spectrometric analyses indicated that one substance was procyanidin B-3 and the other was (+)-catechin (Fig. 9). HPLC analysis revealed the retention time of these two substances to be identical to the authentic standards of procyanidin B-3 and (+)-catechin, respectively. Next, we examined the hair-growing activity of purified procyanidin B-3 and (+)-catechin, with respect to whether they had the ability to stimulate anagen induction using the *in vivo* C3H mouse model. After 19-day application of the

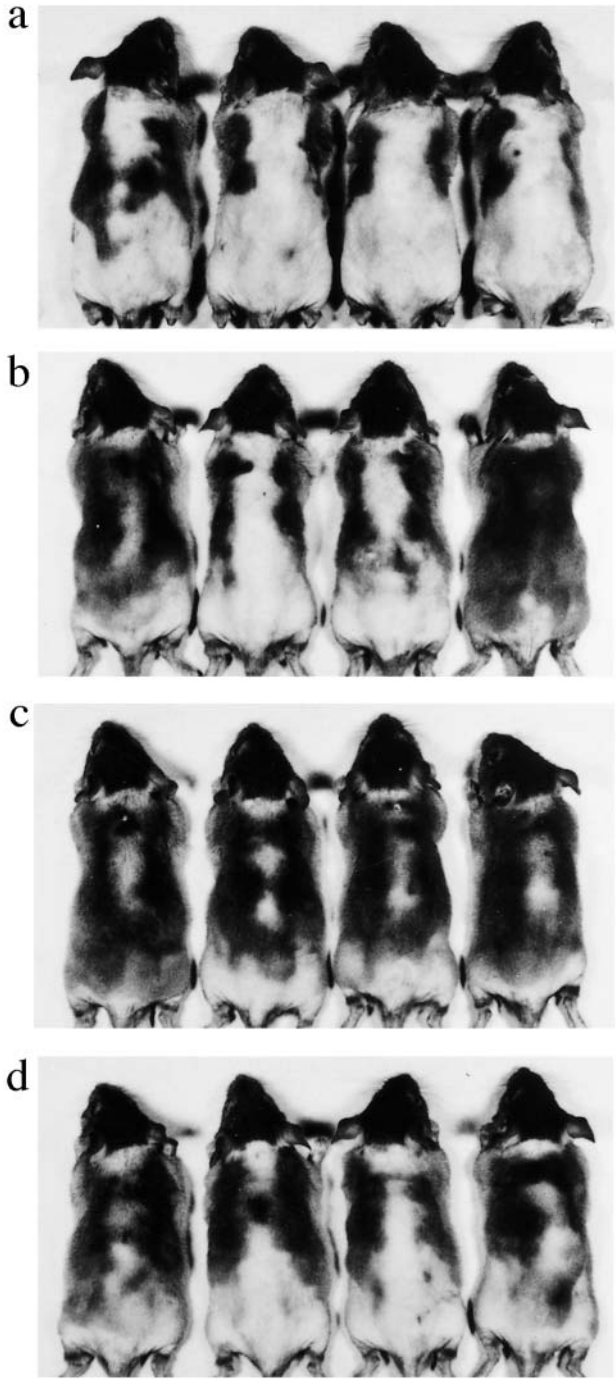

Figure 6. Sixty percent (v/v) aqueous methanol-eluted fraction (Fraction C) from the second HP-20 column intensively induced the anagen phase of the hair cycle in the murine model. Photographs were taken after the topical application of test agents for 19 days. Test agents were applied to 8-week-old male C3H telogen mice at 200 µl/day/mouse. (a) Vehicle, (b) 6% (w/w) Fraction B eluted from second HP-20 column, (c) 6% (w/w) Fraction C eluted from second HP-20 column and (d) 6% (w/w) Fraction D eluted from second HP-20 column.

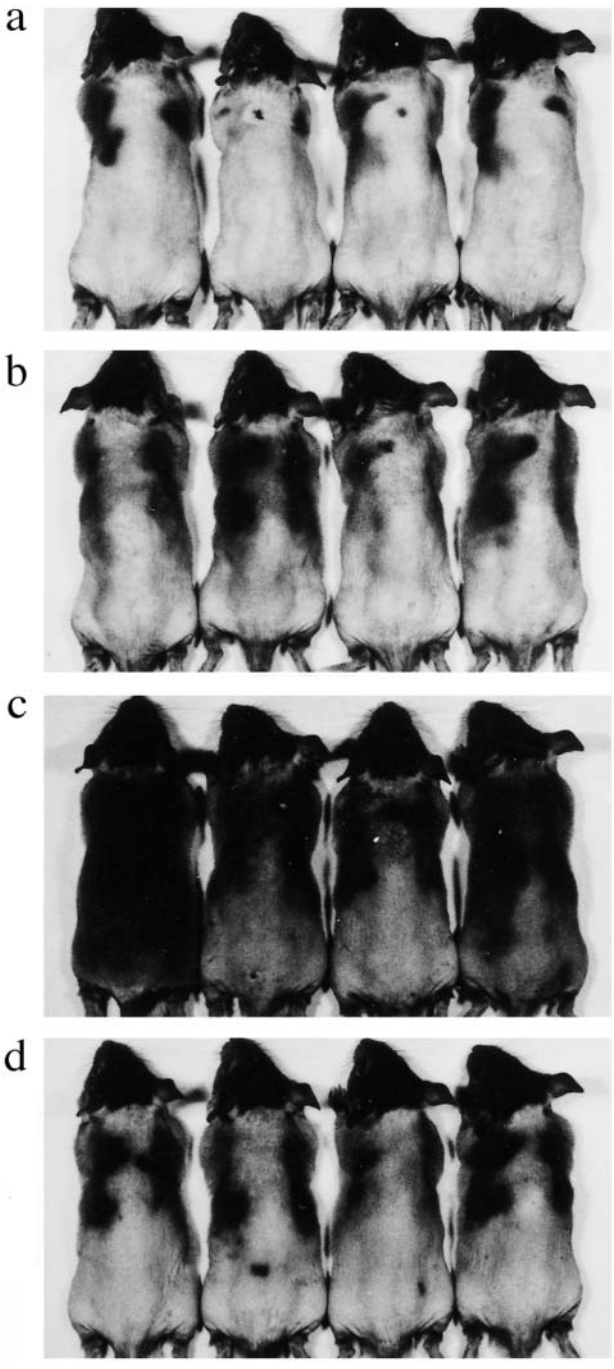

Figure 7. Seventy-five percent (v/v) aqueous methanol-eluted fraction (Fraction F) from an LH-20 column intensively induced the anagen phase of the hair cycle in the murine model. Photographs were taken after the topical application of test agents for 19 days. Test agents were applied to 8-week-old male C3H telogen mice at 200 µl/day/mouse. (a) Vehicle, (b) 3% (w/w) Fraction E eluted from LH-20 column, (c) 3% (w/w) Fraction F eluted from LH-20 column and (d) 3% (w/w) Fraction G eluted from LH-20 column.

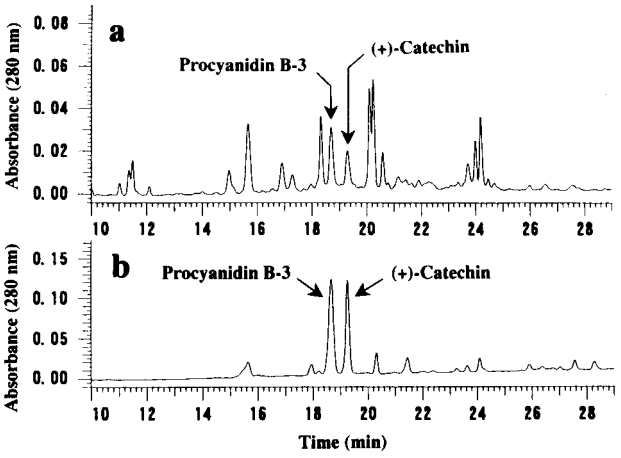

Figure 8. Chromatograms from high-performance liquid chromatography (HPLC) analysis of (a) barley extract and (b) active Fraction F eluted from an LH-20 column. The chromatogram was monitored using a UV detector at the wavelength of 280 nm. A Hitachi L-7000 HPLC system (Hitachi Seisakusho Co., Tokyo, Japan) with an ODS-2 column (5 μm particle size, 4.6 mmϕ × 250 mm, GL Science Co., Tokyo, Japan) was used for the HPLC analysis. The eluting conditions were as follows: flow rate, 1 ml/min; oven temperature, 40°C; mobile phase A = 0.05% (v/v) TFA, mobile phase B = acetonitrile; linear gradients from 0 to 60% (v/v) of B up to 60 min

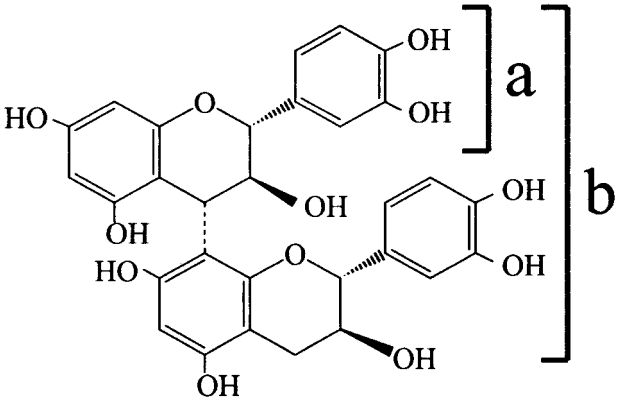

Figure 9. The structures of (a) (+)-catechin and (b) procyanidin B-3 [(+)-catechin-(4αr→8)-(+)-catechin].

agent containing 1% (w/w) procyanidin B-3 to telogen C3H mice, about 80% (80.9% ± 13.0%, average ± SD) of the shaven area was covered with hair; on the other hand, the control group, to which vehicle was applied, showed little hair growth: only about 40% (41.7% ± 16.3%) of the shaven area was covered with hair on Day 19. After application of the agent containing 3% (w/w) (+)-catechin, a flavan-3-ol unit of procyanidin B-3, stimulated no hair growth: only about 40% (38.0% ± 10.8%) of the shaven area of mice was covered with hair on Day 19 (Figs 5 and 10). In experiments to investigate the *in vitro* growth-promoting activity on hair epithelial cells, procyanidin B-3 showed excellent growth-promoting activity of about 300% relative to controls at the optimum concentration of 30 μM (data not shown). These results demonstrate that procyanidin B-3 is the active compound contained in barley extract, which shows growth-promoting activity with respect to hair epithelial cells and hair-growing activity that induces the anagen phase of the hair cycle.

TGF-β1 dose-dependently decreases hair epithelial cell growth, and addition of procyanidin B-3 to the culture counteracts the inhibitory effect of TGF-β1

TGF-β1 is regarded as the likeliest candidate that regulates catagen induction of the hair cycle in *in vivo* mice hair cycle (38). We examined the effect of TGF-β1 on murine hair epithelial cell growth and observed that addition of TGF-β1 to the culture medium dose-dependently inhibits the growth of murine hair epithelial cells. Addition of 10 μM of procyanidin B-3 to medium containing 0.3 ng/ml TGF-β1 completely counteracted the inhibitory effect of TGF-β1 (Fig. 11).

Discussion

Proanthocyanidins

After repeated purification of barley-derived fractions and measurement of their hair-growing activity *in vivo*, we finally identified the active ingredient as procyanidin B-3, one species of proanthocyanidin.

Proanthocyanidins are a species of phenolic compounds highly prevalent in plants; they take

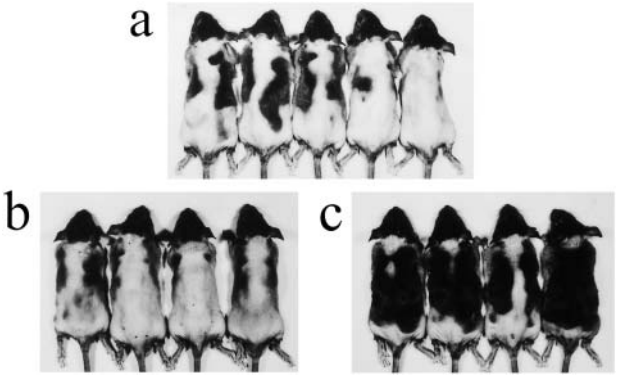

Figure 10. Procyanidin B-3 is able to induce the anagen phase efficiently in hair cycle progression in the murine model; however (+)-catechin did not. Photographs were taken after the topical application of test agents for 19 days. Test agents were applied to 8-week-old male C3H telogen mice at 200 μl/day/mouse. (a) Vehicle, (b) 3% (w/w) (+)-catechin and (c) 1% (w/w) procyanidin B-3.

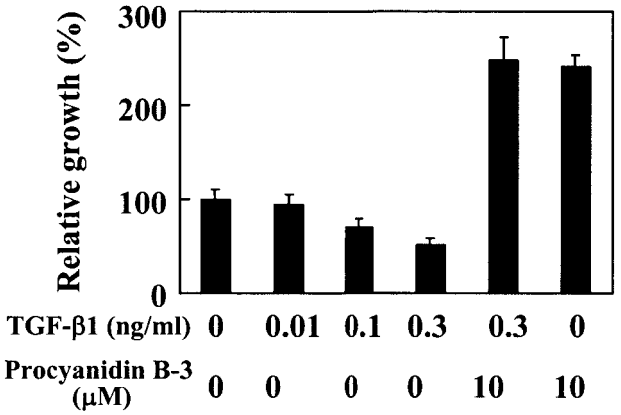

Figure 11. TGF-β1 dose-dependently represses hair epithelial cell growth. Addition of 10 μM procyanidin B-3 counteracts the inhibitory effect of TGF-β1. Growth-promoting activities on hair epithelial cells relative to controls (= 100%) are shown. TGF-β1 and procyanidin B-3 were added to the culture during the last 5 days. For the control, a medium without TGF-β1 or procyanidin B-3 was used. Results are represented as the mean ± SD ($n = 6$) carried out with primary cultures prepared from 50 neonatal mice.

the form of polymers or oligomers built of flavan-3-ol units (39). Proanthocyanidins have long been used in medications and cosmetics designated to protect the capillaries (40) and skin (41). Numerous physiological and pharmacological effects are reported for proanthocyanidins, of which radical scavenging (42), antioxidant activity (43), antihypertensive activity (44), capillary protective action (45), and elastase-inhibiting action (46) are significant from the dermatological viewpoint. However, except for our previous reports describing proanthocyanidins obtained from grape seeds and apples (29, 34), there have been no reports on the hair-growing activity of proanthocyanidins.

Barley-derived proanthocyanidins characteristically contain prodelphinidins and procyanidins. Prodelphinidin is a class of proanthocyanidins whose monomer units are gallocatechin or epigallocatechin, and procyanidin is a class of proanthocyanidins whose monomer units are catechin or epicatechin. Thin-layer chromatography and HPLC analyses comparing with reported results (47–49) have revealed Fraction B to be a prodelphinidin-rich fraction; Fraction C was revealed to be a procyanidin-rich fraction, Fraction F to be a procyanidin oligomer-rich fraction, and Fraction G to be a procyanidin polymer-rich fraction (Table 1). Our experiments revealed the hair-growing activity of prodelphinidins (abundant in Fraction B) to be weaker than that of procyanidins (abundant in Fraction C) (Figs 5 and 6); and the hair-growing activity of procyanidin polymers (abundant in Fraction G) was relatively weaker than that of procyanidin oligomers (abundant in Fraction F) (Figs 5 and 7). On the other hand (+)-catechin, a monomeric unit of procyanidins, showed no hair-growing activity (Figs 5 and 10).

Table 1. The content of proanthocyanidins in each fraction of barley extract

Fraction	Proanthocyanidin content*	Characteristics
Fraction A	19.2%	
Fraction B	57.9%	Prodelphinidin-rich fraction
Fraction C	29.9%	Procyanidin-rich fraction
Fraction D	not detected	
Fraction E	1.8%	
Fraction F	80.0%	Procyanidin oligomer-rich fraction
Fraction G	81.0%	Procyanidin polymer-rich fraction

*Estimated by color production in the vanillin reaction carried out in methanol (33).

It is known that the chemical, physiological and pharmacological properties of proanthocyanidins vary according to their degree of polymerization and the number of hydroxyl groups per molecule. However, very little information has been gathered on the correlation between their degree of polymerization and their properties; or on what differences in properties exist between proanthocyanidins with pyrogallol-type B-rings (i.e. prodelphinidins) and these with catechol type B-rings (i.e. procyanidins). Of the many characteristics possessed by proanthocyanidins, their astringent character is important, because astringency induces complex formation with enzymes and substrates (50). Scholz and Rimpler (51) reported that the intensity of astringency peaks at 7 mer in proanthocyanidins. It has also been shown that increasing the molecular weight of proanthocyanidins results in more efficient binding to proteins through non-covalent linkages (50). Concerning the effects of hydroxyl groups on their properties, interactions between polyphenols and proteins are predicted to increase in proportion to the number of hydroxyl groups in the molecule (52), and thus, non-specific binding of procyanidin polymers and prodelphinidins to protein is assumed to be higher than that of procyanidin oligomers; it is also speculated that procyanidin B-3, a procyanidin oligomer, exerts hair-growing activity not via non-specific interaction with cells but via specific interaction with the components of the cells, such as receptors or enzymes in affected cells. Moreover, it is interesting that the specific reactions affected by procyanidin oligomers, rather than the monomer unit of procyanidins, may be associated with hair growth.

Relationship with the action of TGF-β1

Many factors are known to regulate hair growth and hair cycle progression. For example, IGF-1

and HGF are known to actively regulate hair growth; and TGF-β, FGF-5, TNF-α, IL-1α, and IL-1β are known to regulate hair growth negatively (7). We examined the effect of TGF-β1 on murine hair epithelial cell growth and the effects of procyanidin B-3 on TGF-β1 signal-induced growth inhibition. We subsequently observed that TGF-β1 dose-dependently represses hair epithelial cell growth and demonstrated that procyanidin B-3 has the potential to counteract the inhibitory effect of TGF-β1.

Assumed mechanisms of action of procyanidin B-3

Cotsarelis et al. (53) have suggested that hair follicle stem cells reside in the bulge area of the infundibular region of the outer root sheath. For the anagen phase of the hair cycle to initiate, it is thought that a dramatic burst of mitogenesis first occurs in the hair germ in telogen hair follicles (5, 54); and the subsequent processes, i.e. stem cell activation followed by migration downward of the outer root sheath cells, are considered to be essential for the formation of mature hair follicles (6, 55). As possible hair-growing mechanisms of procyanidin B-3, our speculation is as follows: procyanidin B-3 affects the hair germ, switches it to the growth phase by unknown mechanisms, and induces the anagen phase of the hair cycle; in addition, procyanidin B-3 is expected to exert hair-growing activity by promoting outer root sheath cell proliferation and outer root sheath elongation downward, finally contributing to the formation of mature hair follicles. In addition, it is shown that procyanidin B-3 has the potential to overcome the inhibitory effect of TGF-β1 *in vitro*. This result suggests that procyanidin B-3 has the potential to prevent catagen induction of the hair cycle. The hair-growing activity of procyanidin B-3 may depend on more than one of the numerous physiological functions of this compound.

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