Procyanidin Oligomers Selectively and Intensively Promote Proliferation of Mouse Hair Epithelial Cells *In Vitro* and Activate Hair Follicle Growth *In Vivo*[1]

Tomoya Takahashi, Toshikazu Kamiya, Atsuhiro Hasegawa,* and Yoshiharu Yokoo

Tsukuba Research Laboratories, Kyowa Hakko Kogyo, Tsukuba, Ibaraki, Japan; *Tokyo Research Laboratories, Kyowa Hakko Kogyo, Machida, Tokyo, Japan

We have previously reported that proanthocyanidins extracted from grape seeds possess growth-promoting activity toward murine hair epithelial cells *in vitro* and stimulate anagen induction in hair cycle progression *in vivo*. This report constitutes a comparison of the growth-promoting activity of procyanidin oligomers and the target cells of procyanidins in the skin. Results show that procyanidin dimer and trimer exhibit higher growth-promoting activity than the monomer. The maximum growth-promoting activity for hair epithelial cells with procyanidin B-2, an epicatechin dimer, reached about 300% (30 μM) relative to controls (=100%) in a 5 d culture. Optimum concentration of procyanidin C-1, an epicatechin trimer, was lower than that of procyanidin B-2; the maximum growth-promoting activity of procyanidin C-1 was about 220% (3 μM). No other flavonoid compounds examined exhibit higher proliferative activities than the procyanidins. In skin constituent cells, only epithelial cells such as hair keratinocytes or epidermal keratinocytes respond to procyanidin oligomers. Topical application of 1% procyanidin oligomers on shaven C3H mice in the telogen phase led to significant hair regeneration [procyanidin B-2, 69.6% ± 21.8% (mean ± SD); procyanidin B-3, 80.9% ± 13.0%; procyanidin C-1, 78.3% ± 7.6%] on the basis of the shaven area; application of vehicle only led to regeneration of 41.7% (SD = 16.3%). In this paper, we demonstrate the hair-growing activity of procyanidin oligomers both *in vitro* and *in vivo,* and their potential for use as agents to induce hair growth. ***Key words: cell culture/condensed tannin/hair growth/skin. J Invest Dermatol 112:310–316, 1999***

Although many plant extracts have been tried for curing male pattern baldness, in most cases the efficacy was uncertain or the active materials were unknown. Minoxidil, which was originally synthesized as a potassium channel opener (Buhl *et al*, 1993) and initially prescribed for hypertension, is an FDA approved topical medication for curing male pattern baldness (Kulick, 1988; Olsen, 1989). Recently, finasteride, which is a type II 5α-reductase inhibitor and was initially used for curing prostatic hypertrophy (Dallob *et al*, 1994; Gormley, 1995), has been approved by the FDA as an oral drug for curing male pattern baldness (Kaufman *et al*, 1998). Several side-effects[2] and one accident have been reported with both agents (McCormic *et al*, 1989; Boeck *et al*, 1996; Nickel *et al*, 1996; Wilton *et al*, 1996). It has been reported that cyclosporine A stimulates anagen induction (Paus *et al*, 1989) and causes hirsutism (Wysocki and Daley, 1987). Capsaicin injected into telogen mice has been reported to cause anagen induction (Paus *et al*, 1994).

Manuscript received July 6, 1998; revised September 3, 1998; accepted for publication November 25, 1998.

Reprint requests to: Dr. Tomoya Takahashi, Tsukuba Research Laboratories, Kyowa Hakko Kogyo Co., 2, Miyukigaoka, Tsukuba, Ibaraki 305-0841, Japan.

[1]The authors disclosed conflict of interest.

[2]Wilson C, Walkden V, Powell S, Shaw S, Wilkinson J, Dawber R: Contact dermatitis in reaction to 2% topical minoxidil solution. *J Am Acad Dermatol* 24:661–662, 1991 (brief communications)

More recently, we have found hair epithelial cell growth-promoting activity and ability to induce anagen phase in proanthocyanidins extracted from grape seeds (Takahashi *et al*, 1998) as an investigation of a wide range of plant extracts numbering more than a thousand. Proanthocyanidins are a species of phenolic compounds that take the form of polymers or oligomers built of flavan-3-ol units, such as catechin, epicatechin, gallocatechin, epigallocatechin, afzelechin, and epiafzelechin; whose molecules occasionally incorporate gallic acid (Porter, 1994). Proanthocyanidins are very common in the plant kingdom. The significance of the existence of proanthocyanidins in plants is considered to be their anti-microbial action, their deterrence properties, and protection from ultraviolet rays (Bernays *et al*, 1989; Scalbert, 1991). Concerning the physiologic and pharmacologic effects of proanthocyanidins, the following activities have been reported: radical scavenging (Vennat *et al*, 1994), anti-oxidant (Hong *et al*, 1995), anti-mutagenic (Liviero *et al*, 1994), anti-tumor-promoting (Gali *et al*, 1994), anti-fungal (Eberhardt and Young, 1994), anti-viral (Barnard *et al*, 1993), anti-ulcer (Vennat *et al*, 1989), anti-hypertensive activity (Cheng *et al*, 1993), and capillary protective action (Facino *et al*, 1994). There are no reports, however, on the hair-growing activity of proanthocyanidins, except for our previous report (Takahashi *et al*, 1998) on proanthocyanidins purified from grape seeds.

We examined which type of proanthocyanidin molecules possess the highest hair-growing activity. We report here that procyanidin oligomers possess selective and intensive growth-promoting activity with respect to hair epithelial cells *in vitro* and stimulate anagen

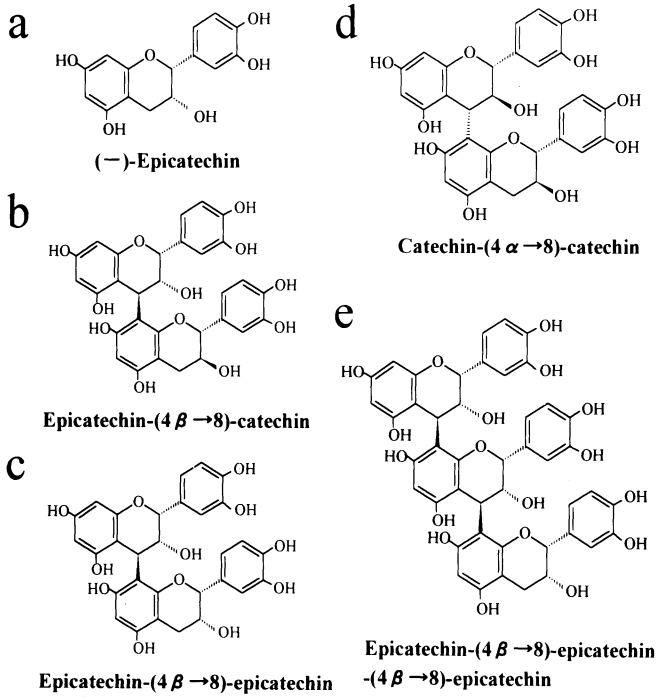

Figure 1. The structures of procyanidin monomer and oligomers. (*a*) (–)-Epicatechin; (*b*), procyanidin B-1; (*c*), procyanidin B-2; (*d*), procyanidin B-3; (*e*), procyanidin C-1.

induction *in vivo*. As the mechanism of action of procyanidins, we expect there to be a direct effect on the follicular epithelium in the skin tissues.

MATERIALS AND METHODS

Materials (–)-Epicatechin (**Fig 1**) and hesperetin were purchased from Sigma (St. Louis, MO). Chrysin, kaempferol (+)-taxifolin, and cyanidin chloride were purchased from Extrasynthese S.A. (Genay, France). Genistein and phloretin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Quercetin was purchased from Nacalai Tesque (Kyoto, Japan).

Isolation of procyanidin B-1 [epicatechin-(4β→8)-catechin] Twenty kiloliters of apple juice (*Malus pumila* Miller var. *domestica* Schneider, Fuji variety, commercial juice) was passed through a column (60 cmϕ × 88.5 cm) filled with Diaion HP-20 resin (Mitsubishi Kasei, Tokyo, Japan), equilibrated with demineralized water, then washed with 1000 liters of demineralized water and 500 liters of 15(vol/vol)% methanol. Five hundred liters of 45(vol/vol)% methanol was applied and the column was then eluted to produce 9450 g of a dry solid. Then, 1465 g of this was passed through a column (18 cmϕ × 39.3 cm) filled with Sephadex LH-20 (Pharmacia, Uppsala, Sweden) equilibrated with 25(vol/vol)% methanol, then washed with 20 liters of 25(vol/vol)% methanol and 20 liters of 50(vol/vol)% methanol. Twenty liters of 75(vol/vol)% methanol was passed through the column and the intended fraction was eluted to produce 233 g of a dry solid. Half of this was subjected to preparative high-performance liquid chromatography (150 mmϕ × 1000 mm, Soken, Japan; column packing was ODS silica gel, 50 μm particle size, KE-ODS-50 SRQ, YMC, Kyoto, Japan). The eluting conditions were as follows: flow-rate, 645 ml per min; room temperature; mobile phase A = 0.0001% acetic acid, mobile phase B = methanol; isocratic elution with A = 88%, B = 12%; monitored by a UV detector at a wavelength of 285 nm. A total of 107 g of procyanidin B-1 of purity greater than 94(wt/wt)% in dry weight was obtained from 20 kl of apple juice. The product was identified by mass spectrometry, ^{1}H-NMR, and ^{13}C-NMR (Thompson *et al*, 1972; Nonaka *et al*, 1981).

Isolation of procyanidin B-2 [epicatechin-(4β→8)-epicatechin] Procyanidin B-2 (**Fig 1**) was isolated using the same procedure as previously mentioned for procyanidin B-1 purification: namely, 213 g of procyanidin B-2 was obtained from 20 kl of apple juice. The product was identified by mass spectrometry, ^{1}H-NMR, and ^{13}C-NMR (Thompson *et al*, 1972; Morimoto *et al*, 1986).

Isolation of procyanidin C-1 [epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin] Procyanidin C-1 (**Fig 1**) was isolated using the same procedure as previously described for procyanidin B-1 purification: namely, 21 g of procyanidin C-1 was obtained from 20 kl of apple juice. The product was identified by mass spectrometry, ^{1}H-NMR, and ^{13}C-NMR (Nonaka *et al*, 1982; Morimoto *et al*, 1986).

Isolation of procyanidin B-3 [catechin-(4α→8)-catechin] Procyanidin B-3 (**Fig 1**) was isolated from barley grains as follows: 60 kg barley grains (*Hordeum vulgare* L. var. *distichon* Alefeld) was extracted with 90 kg of 70(wt/wt)% acetone at room temperature for 4 d. The extract was filtered through filter paper (No. 526, Advantec Toyo, Tokyo, Japan) to obtain an extract. The extract was then evaporated and passed through an HP-20 column (10 cmϕ × 50 cm size with a volume of 3925 ml) that had been equilibrated with demineralized water. After washing the column with 8 liters of 20(vol/vol)% methanol, 8 liters of 40(vol/vol)% methanol was applied to the column. The intended fraction was eluted to produce 23.4 g of a dry solid. The dry solid was dissolved in distilled water and then subjected to preparative high-performance liquid chromatography (ODS column, 15 μm particle size, 3 cmϕ × 30 cm, Nihon Waters, Tokyo, Japan) to obtain 1.4 g of procyanidin B-3. The product was identified by mass spectrometry, ^{1}H-NMR, and ^{13}C-NMR (Thompson *et al*, 1972; Brandon *et al*, 1982).

Isolation and culturing of hair epithelial cells Mouse hair epithelial cells were isolated and cultured according to the method reported by Tanigaki *et al* (1990) with suitable modifications. The dorsal skin was peeled from 4 d old C3H/HeNCrj mice (Charles River Japan, Kanagawa, Japan), cut into about 5 mm widths, then dipped into Eagle's minimum essential medium containing 500 IU dispase per ml (from *Bacillus polymyxa*, Godo Shusei, Tokyo, Japan), 60 μg kanamycin per ml, and 5% fetal calf serum at 4°C for 16 h. The epidermis was peeled off, and the remaining dermis layer was dispersed with Dulbecco's modified Eagle medium (DMEM) containing 0.25% collagenase (from *Streptomyces parvulus*, Nitta Gelatin, Osaka, Japan), 50 U penicillin per ml, 50 μg streptomycin per ml, and 10% fetal calf serum at 37°C for 1 h. This dermis suspension was filtered through a 212 μm nylon mesh, and the filtrate was centrifuged at 1000 r.p.m. for 5 min. The pellet was suspended in phosphate-buffered saline without calcium and magnesium. The suspension was left to stand for 15 min, whereby the hair follicle tissue was precipitated. The hair follicle tissue was resuspended in phosphate-buffered saline without calcium and magnesium and then precipitated. This precipitation process was repeated three times. The hair follicle tissue was finally incubated in 0.05% EDTA-0.25% trypsin at 37°C for 5 min. The hair follicle cells were suspended in DMEM supplemented with 50 U penicillin per ml, 50 μg streptomycin per ml, and 10% fetal calf serum at the density of 3×10^5 cells per ml. This hair follicle cell suspension was pipetted into a 24 well Type I collagen-coated plate (2 cm^2 per well, Iwaki Glass, Chiba, Japan) with 1 ml per well and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 24 h. After 24 h incubation, the medium was exchanged with MCDB153 (Kyokuto Pharmaceutical, Tokyo, Japan) (Boyce and Ham, 1983) containing 5 mg bovine insulin per liter, 5 μg mouse EGF per liter, 40 mg bovine pituitary extract per liter, 10 mg human transferrin per liter, 0.4 mg hydrocortisone per liter, 0.63 μg progesterone per liter, 14 mg *O*-phosphorylethanolamine per liter, 6.1 mg ethanolamine per liter, 50 U penicillin per ml, and 50 μg streptomycin per ml. It was then further incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 5 d. During incubation, the medium was removed and replaced with fresh medium every other day.

Isolation and culturing of dermal papilla cells from rat vibrissae Dermal papillae were isolated from PVG/Sea rat (10 wk old, male, purchased from Japan SLC, Shizuoka, Japan; breeding by Seiwa Experimental Animal Research Laboratory, Fukuoka, Japan) vibrissa follicles according to the method of Jahoda and Oliver (1981). The rats were anesthetized and the whisker follicles were dissected. The lower halves of the follicle were sectioned and placed into DMEM containing 50 U penicillin per ml, 50 μg streptomycin per ml, and 20% fetal calf serum. For the following procedure, we used a needle (24G × 1″, 0.55 × 25 mm, Terumo, Tokyo, Japan) and scalpel (Mini no. 15 disposable scalpel, Feather, Osaka, Japan) for the isolation of dermal papillae from vibrissa follicles. Under a stereoscopic microscope, holding the vibrissa follicle by the needle, we cut horizontally just above the hair bulb with the scalpel, and pulled out the hair shaft using a pair of tweezers. Then, holding the hair bulb with the needle, we peeled and turned over the outer root sheath around the dermal papilla using another needle from the upper side. The exposed dermal papillae were sectioned by needle and transferred on a needle tip

to the 6 well culture plate (9.6 cm^2 per well, Falcon no. 3046, Nippon Becton Dickinson, Tokyo, Japan). After rubbing the dermal papilla onto the culture plate and leaving it to stand for cultivation in DMEM containing 50 U penicillin per ml, 50 μg streptomycin per ml, and 20% fetal calf serum, dermal papilla cells grew out from the original sections. After 2 wk of culture, the culture plate became subconfluent, and 0.02% EDTA-0.1% trypsin treatment was carried out. By adding DMEM containing 50 U penicillin per ml, 50 μg streptomycin per ml, and 20% fetal calf serum, a cell suspension containing 3×10^4 cells per ml was prepared. The dermal papilla cell suspension was pipetted into a 24 well culture plate (2 cm^2 per well, Falcon no. 3047, Nippon Becton Dickinson) with 1 ml per well and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 7 d. During incubation, the medium was removed and replaced with fresh medium every other day.

Isolation and culturing of mouse epidermal keratinocytes Keratinocytes were obtained from the EDTA-trypsin treated epidermis of C3H/HeNCrj mouse and cultured in a 24 well collagen Type I coated plate (at the initial cell density of 5×10^4 cells per cm^2, Iwaki Glass, Japan) in the same manner described at hair epithelial cells using an DMEM for the first 24 h and MCDB153 (containing the same additives as that of the hair epithelial cells) for the next 6 d.

Isolation and culturing of mouse dermal fibroblasts Dermal fibroblasts were obtained from the EDTA-trypsin treated dermis of C3H/HeNCrj mouse and cultured in a 24 well culture plate (at the initial cell density of 1.5×10^4 cells per cm^2, Falcon no. 3047) using a DMEM (supplemented with 50 U penicillin per ml, 50 μg of streptomycin per ml, and 10% fetal calf serum) in a humidified atmosphere containing 5% CO_2 at 37°C for 5 d.

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 (Carmichael *et al*, 1987). To summarize, MTT reagent (5 mg per ml, dissolved in water and filtered through a 0.2 μm membrane filter) was added by a volume of 1/100 to the culture medium and 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 0.1 N HCl (adding 0.4 ml per 2 cm^2 of well), and the absorbance was measured at 570 nm relative to 640 nm.

Preparation methods of topically applied agents for *in vivo* evaluation Fourteen grams of ethyl alcohol, 0.2 g of procyanidin oligomers, 2 g of 1,3-butylene glycol, 0.1 g of *N*-acetylglutamine-isostearyl (Kyowa Hakko Kogyo, Tokyo, Japan), 0.05 g of polyoxyethylene (25) glyceryl monopyroglutamate monoisostearate (Nihon Emulsion, Tokyo, Japan), and 3.65 g of pure water were mixed together, and the solids were dissolved to prepare a sample solution for the *in vivo* mice test.

A vehicle without procyanidins was used as the control. Minoxidil (Upjohn Company, Kalamazoo, MI; purchased from Sigma) agent was prepared in the same way as the procyanidin-containing agent.

Test for hair-growing activity to induce anagen phase in mice With reference to the method introduced by Hattori and Ogawa (1983), the hair-growing activity to induce anagen phase in mice was measured. In this test, 8 wk old male C3H/HeS1c (Japan SLC, Shizuoka, Japan) mice whose hair cycle was in the telogen stage were used. The mice were grouped into multiple groups; each one comprised of 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. (Otherwise stimulation would raise background activity for control.) Two hundred microliters of test sample was applied onto the shaven area once a day. On the nineteenth day of the test, the mouse back skins were observed and photographed; then the skin was peeled from the back of each mouse and photographed.

RESULTS

Procyanidin dimer and trimer selectively and intensively promote growth of hair epithelial cells We compared the growth-promoting activity on hair epithelial cells by procyanidins of different degrees of polymerization, and of different flavan-3-ol units: catechin, epicatechin, or a combination of both. For the degree of polymerization, we compared the growth-promoting activity of the monomer, dimer, and trimer: we report on the example of (–)-epicatechin, procyanidin B-2 [epicatechin-(4β→8)-epicatechin], and procyanidin C-1 [epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin] (**Fig 2**). For the combination of flavan-3-ol units, we report on procyanidin B-1 [epicatechin-(4β→8)-catechin], procyanidin B-2 [epicatechin-(4β→8)-epicatechin], and procyanidin B-3 [catechin-(4α→8)-catechin] (**Fig 3**).

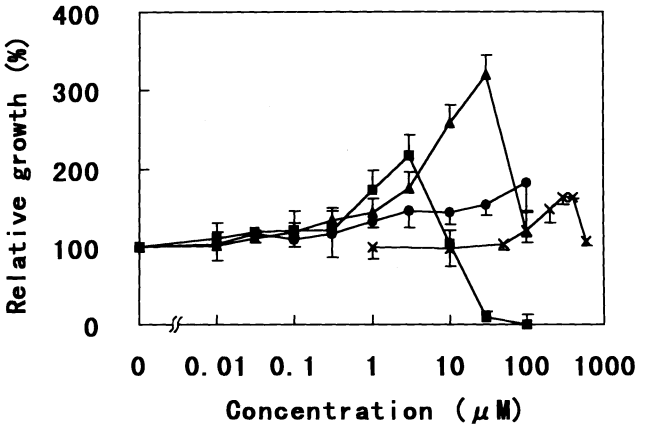

Figure 2. Procyanidin dimer and trimer more intensively promote growth of hair epithelial cells than the procyanidin monomer. Growth-promoting activities for hair epithelial cells relative to controls (=100%) are shown. Procyanidins were added to the culture during the last 5 d. For the control, a medium without procyanidins was used. ●, (–)-Epicatechin; ▲, procyanidin B-2; ■, procyanidin C-1; ×, minoxidil. Results are represented as the mean ± SD (n = 6) carried out with primary cultures prepared from 50 neonatal mice.

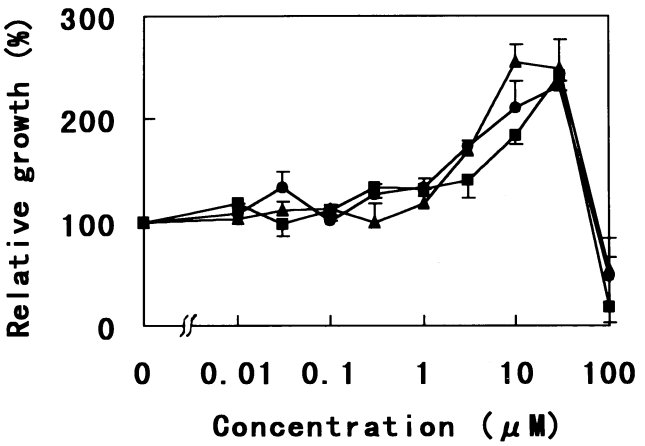

Figure 3. Procyanidin dimeric isomers with different flavan-3-ol units also promote the growth of hair epithelial cells. Growth-promoting activities for hair epithelial cells relative to controls (=100%) are shown. Procyanidins were added to the culture during the last 5 d. For the control, a medium without procyanidins was used. ●, Procyanidin B-1; ▲, procyanidin B-2; ■, procyanidin B-3. Results are represented as the mean ± SD (n = 6) carried out with primary cultures prepared from 50 neonatal mice.

Of (–)-epicatechin, procyanidin B-2, and procyanidin C-1, procyanidin B-2 shows the highest growth-promoting activity of about 300% relative to controls (= 100%) in a 5 d culture of hair epithelial cells. The optimum concentration of procyanidin C-1 was lower than that of procyanidin B-2, but the maximum growth-promoting activity of procyanidin C-1 relative to controls (=100%) was about 220%, lower than that of procyanidin B-2; however, at concentrations from 0.3–3 μM, the growth-promoting activity of procyanidin C-1 was higher than that of procyanidin B-2. The activity of (–)-epicatechin was lower than those of procyanidin B-2 and procyanidin C-1 (**Fig 2**). No significant difference in growth-promoting activity was observed among procyanidin B-1, procyanidin B-2, and procyanidin B-3 (**Fig 3**); their maximum growth-promoting activities were higher than those of the monomer or trimer of procyanidins. On the other

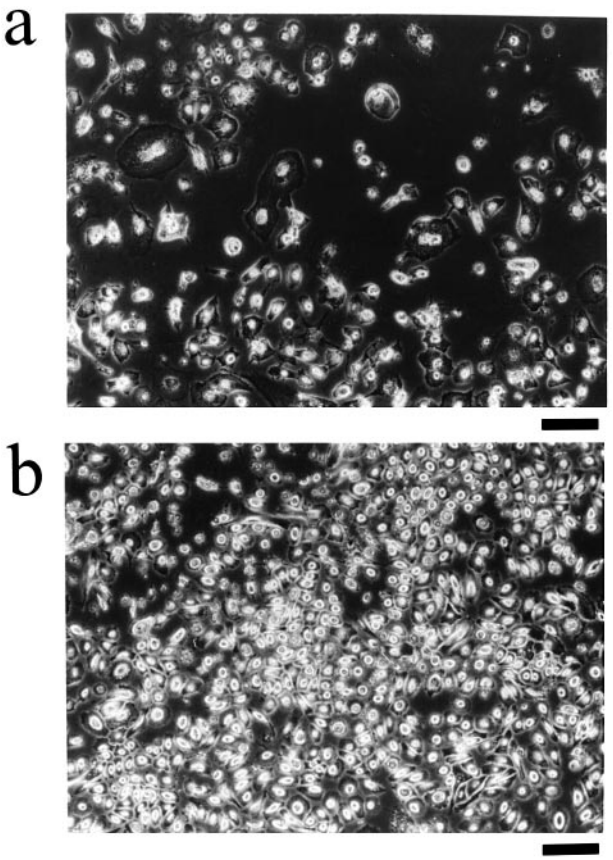

Figure 4. Micrographs of hair epithelial cells from mice cultured in MCDB-153 medium for 5 d. (*a*) Control, (*b*) 30 μM procyanidin B-2. *Scale bar.* 100 μm.

hand, minoxidil was less effective in this cell culture system, demonstrating about 160% proliferative activity at a concentration of 400 μM (**Fig 2**). The micrograph of hair epithelial cells from mice cultured in procyanidin B-2 containing medium is shown in **Fig 4**.

We compared the growth-promoting activity of procyanidins on hair epithelial cells with the activities of other flavonoid compounds. Among flavonoids, we selected typical compounds from each group: flavones, isoflavones, flavonols, flavanones, dihydroflavonols, dihydrochalcones, and anthocyanidins. From flavones we chose chrisin; from isoflavones, genistein; from flavonols, kaempferol and quercetin; from flavanones, hesperetin; from dihydroflavonols, taxifolin; from dihydrochalcones, phloretin; and from anthocyanidins, cyanidin chloride. **Figure 5** shows the growth-promoting activity on hair epithelial cells by these flavonoid compounds. Among the examined flavonoid compounds, procyanidin was the only compound that showed a high level of growth-promoting activity on hair epithelial cells: greater than 300% (procyanidin B-2).

On the other hand, procyanidins did not affect the proliferation of dermal papilla cells (**Fig 6**).

Procyanidin dimer and trimer selectively and intensively promote growth of mouse keratinocytes We examined the effect of procyanidins on both types of skin cells: epithelial cells and mesenchymal cells. Specifically, we examined the effect of procyanidins on the proliferation of keratinocytes and fibroblasts.

Figure 7 shows the effect of procyanidins on keratinocyte cell growth. Among (–)-epicatechin, procyanidin B-2, and procyanidin C-1, procyanidin B-2 showed the highest growth-promoting activity of about 250% relative to controls (=100%) at concentrations of 0.3–100 μM. The growth-promoting activity of procyanidin C-1 was about 210% relative to controls (=100%), lower than

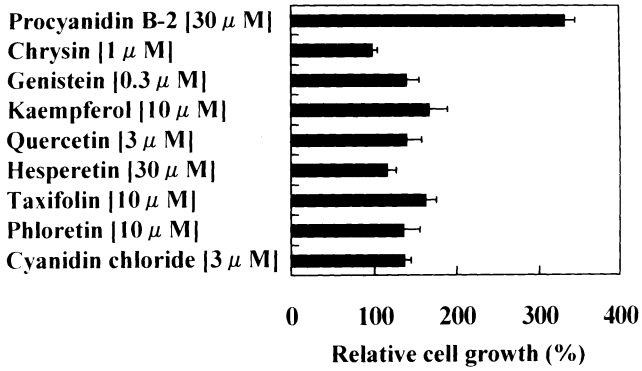

Figure 5. Procyanidins selectively promote the growth of hair epithelial cells. Other flavonoid compounds examined, however, only slightly stimulate the growth of hair epithelial cells. Maximum growth-promoting activities relative to controls (=100%) are shown. Flavonoid compounds were added to the culture during the last 5 d. For the control, we used a medium to which an amount of 1/100 volume of dimethyl sulfoxide (DMSO) (for chrisin, genistein, kaempferol, quercetin, hesperetin, taxifolin, phloretin, and cyanidin chloride) or water (for procyanidin B-2) was added in place of flavonoid containing solution. We observed no significant difference between DMSO control and water control. Brackets show optimum concentration. Results are represented as the mean ± SD (n = 6) carried out with primary cultures prepared from 50 neonatal mice.

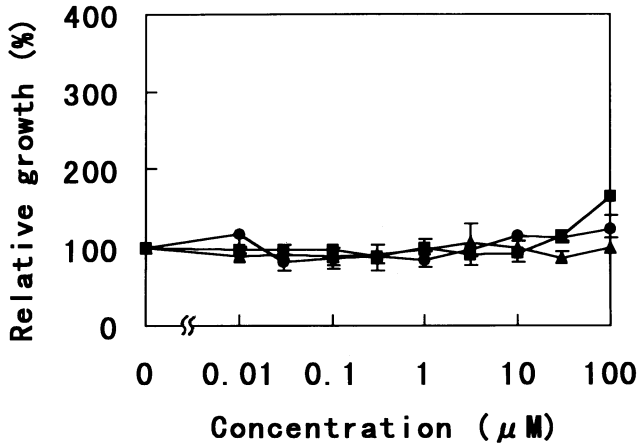

Figure 6. Dermal papilla cells did not respond in cell growth by procyanidins with different degrees of polymerization. Growth-promoting activities for dermal papilla cells relative to controls (=100%) are shown. Procyanidins were added to the culture during the last 6 d. For the control, a medium without procyanidins was used. ●, (–)-Epicatechin; ▲, procyanidin B-2; ■, procyanidin C-1. Results are represented as the mean ± SD (n = 6) carried out with secondary cultures prepared from 20 follicles of two rats.

that of procyanidin B-2, at concentrations of 0.1–10 μM. The optimum concentration of procyanidin C-1 was lower than that of procyanidin B-2. The activity of (–)-epicatechin was relatively lower than the activities of procyanidin B-2 and procyanidin C-1. This proliferative pattern, related to the degree of polymerization of procyanidins, resembles that of hair epithelial cells.

On the other hand, dermal fibroblast growth was not affected by the procyanidins examined (**Fig 8**).

Procyanidin oligomers stimulate anagen induction in hair cycle progression in murine model C3H mouse dorsal hair is known to have a time-synchronized hair cycle. From about 18–21 d of age and 47–95 d of age, the dorsal hairs are in the telogen phase (Hattori and Ogawa, 1983). The test compound was topically applied from the eighth to the tenth week (19 d application) during the second telogen phase, and the hair-covered area at the tenth week was evaluated.

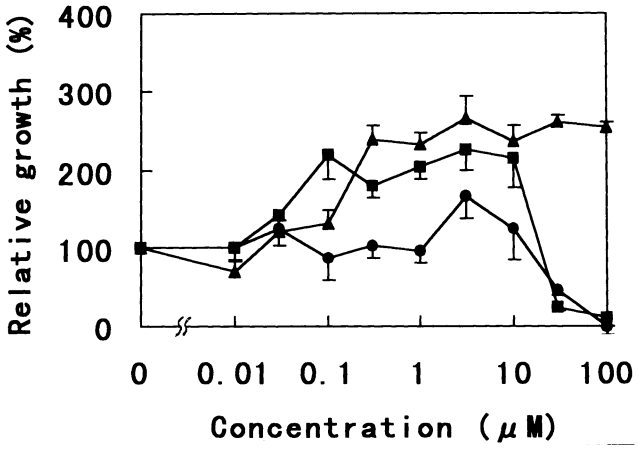

Figure 7. Procyanidin dimer and trimer intensively promote the growth of mouse keratinocytes to a greater extent than the procyanidin monomer. Growth-promoting activities for keratinocytes relative to controls (=100%) are shown. Procyanidins were added to the culture during the last 6 d. For the control, a medium without procyanidins was used. ●, (–)-Epicatechin; ▲, procyanidin B-2; ■, procyanidin C-1. Results are represented as the mean ± SD (n = 6) carried out with primary cultures prepared from 50 neonatal mice.

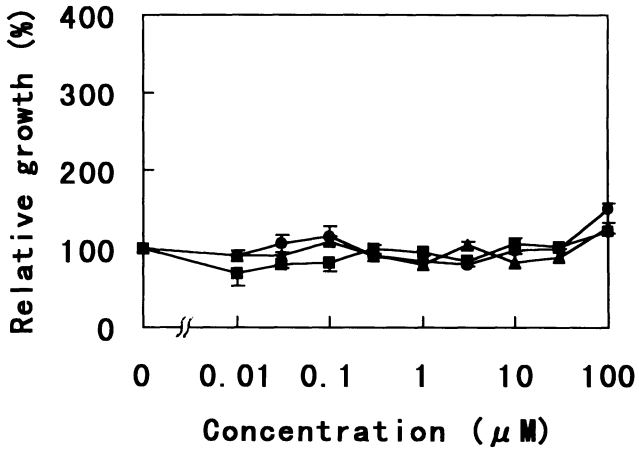

Figure 8. Dermal fibroblasts did not respond when exposed to procyanidins with different degrees of polymerization. Growth-promoting activities for dermal fibroblasts relative to controls (=100%) are shown. Procyanidins were added to the culture during the last 4 d. For the control, a medium without procyanidins was used. ●, (–)-Epicatechin; ▲, procyanidin B-2; ■, procyanidin C-1. Results are represented as the mean ± SD (n = 6) carried out with primary cultures prepared from 50 neonatal mice.

In this assay system, minoxidil gave a positive response. After a 19 d application of 1% minoxidil-containing agent, about 80% (81.2% ± 10.5%, average ± SD) of the shaven area was covered with hair. The control group to which vehicle was applied, on the other hand, showed little hair growth: only about 40% (41.7% ± 16.3%) of the shaven area was covered with hair on day 19. The groups to which 1% procyanidin oligomers had been applied showed an extensive growth area [procyanidin B-2, 69.6% ± 21.8% (average ± SD); procyanidin B-3, 80.9% ± 13.0%; procyanidin C-1, 78.3% ± 7.6%] on day 19 (**Fig 9**).

These results demonstrate that procyanidin oligomers, such as procyanidin B-2, procyanidin B-3, or procyanidin C-1, possess marked hair-growing activity to induce anagen phase *in vivo*. On the other hand, neither (+)-catechin nor (–)-epicatechin, a flavan-3-ol unit of procyanidins, stimulated anagen induction *in vivo* (data not shown).

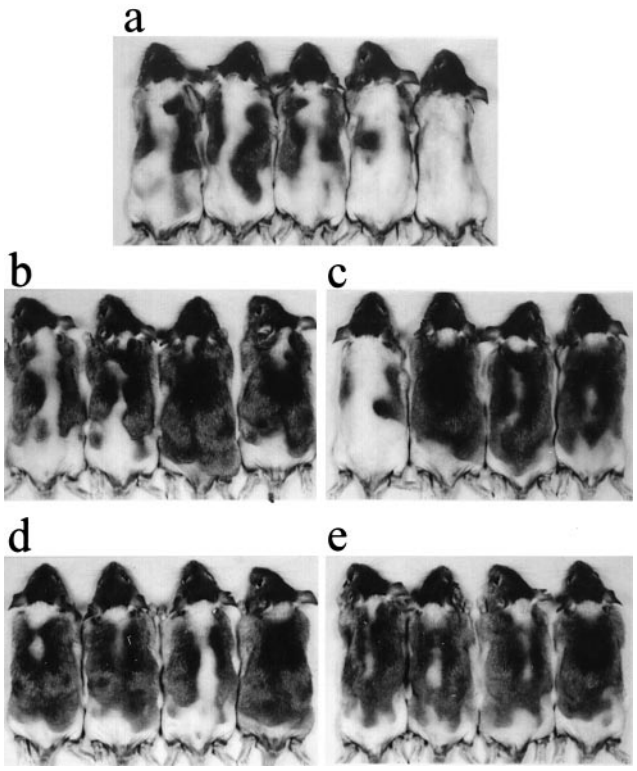

Figure 9. Procyanidin oligomers are able to induce anagen phase efficiently in hair cycle progression in the murine model to the same degree as minoxidil. Photographs were taken after the topical application of test agents for 19 d. Test agents were applied to 8 wk old C3H telogen mice (♂), 200 μl per d per mouse. (*a*) Vehicle; (*b*) 1% minoxidil; (*c*) 1% procyanidin B-2; (*d*) 1% procyanidin B-3; (*e*) 1% procyanidin C-1.

DISCUSSION

Proanthocyanidins Proanthocyanidins have been used as medications aimed at protecting the capillary vessels (Dartenuc *et al*, 1980), as cosmetics to protect the skin (Wayne *et al*, 1996), and as antioxidants in foods and beverages; however, there has been very little information gathered on the correlation between their degree of polymerization and properties. Barnard *et al* (1993) report that proanthocyanidin polymers possess anti-viral activities against the herpes virus. Ariga *et al* (1988) report that its anti-oxidant properties increase in proportion to the degree of polymerization. The profile of the proanthocyanidins from grape seeds in which we first discovered their hair-growing activity is as follows: the constitutive monomers were catechin and epicatechin, the degree of polymerization was 3.5, and the galloylation rate was 25% at the molar ratio per constitutive flavan-3-ol unit (Takahashi *et al*, 1998). Here, we investigated which proanthocyanidin molecules possess the highest hair-growing activity. It was revealed that among the monomer, dimer, and trimer of procyanidins, procyanidin dimer possesses the highest growth-promoting activity. The order of optimum concentration showing the maximum proliferative activity was trimer < dimer < monomer. In *in vivo* hair-growing activity to induce anagen phase, the intensity of dimers and trimers was almost equal; however, neither (–)-epicatechin nor (+)-catechin, monomers of proanthocyanidins, stimulated anagen induction. In *in vitro* and *in vivo* assays, we could find no difference between dimeric isomers composed of different monomers: procyanidin B-1, procyanidin B-2, and procyanidin B-3. The excision of gallate using tannase from proanthocyanidins purified from grape seeds raises the activity *in vitro* and *in vivo* (data not shown). Our *in vitro* studies led to the discovery of no other active flavonoid compounds. The procyanidin compounds we examined were built only of flavan-3-ol, so hair-growing activity may be associated with this structure.

Target cells Skin is composed of keratinocytes, which are epithelial cells, and fibroblasts, which are mesenchymal cells. Hair follicles consist of the outer root sheath, the inner root sheath, and the bulb matrix, which are epithelial cells, and the dermal papillae, which are mesenchymal cells. It is thought that the interaction between epithelial cells and mesenchymal cells is important in the progress of the hair cycle. It is reported that androgen receptors exist in the dermal papilla (Hibberts *et al*, 1998); it is speculated that secretion of several growth factors and cytokines from the hair follicle, such as the dermal papilla, the outer root sheath, the inner root sheath, or the bulb matrix, affect epidermal cell growth at the bulb region, and promote the hair cycle (Messenger, 1993; Danilenko *et al*, 1996). In this paper, we have demonstrated that procyanidin oligomers directly promote the proliferation of the hair follicular epithelium and do not affect dermal papilla cells at least in proliferation level. On the other hand, we also demonstrated that procyanidin oligomers promote the proliferation of keratinocytes in skin. From this, we conclude that procyanidins directly affect the growth of the epithelial cells in skin. Interestingly, the pattern of proliferative activity of keratinocytes in relation to the polymerization degree of procyanidins was similar to that for hair epithelial cells (**Figs 2**, **7**).

Assumed mechanism of action of procyanidins It is known that minoxidil causes the proliferation of hair epithelial cells *in vitro* (Tanigaki-Obana and Ito, 1992; Buhl *et al*, 1989). We have illustrated that procyanidin oligomers possess high growth-promoting activity on hair keratinocytes and epidermal keratinocytes, which are classified as epithelial cells. It is noteworthy that the morphology of hair epithelial cells cultured in a procyanidin-containing medium takes on the rounded appearance characteristic of undifferentiated juvenile cells (**Fig 4**).

Stem cell existence was assumed at the bulge area of the infundibular region of the outer root sheath by Cotsarelis *et al* (1990). So, the outer root sheath appears to be important in the progress of the hair cycle. It is assumed that the growth-promoting effects of procyanidins 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. We are now investigating the relationship between protein kinase C-inhibiting activity by procyanidins (Kashiwada *et al*, 1992; Cui *et al*, 1993; Polya and Foo, 1994) and hair growing activity.

Potential for the use of procyanidins as agents inducing hair growth We have demonstrated that procyanidin oligomers possess both hair epithelial cell growth-promoting activity and hair-growing activity to induce anagen phase *in vivo*. The mechanism of androgenetic alopecia is now speculative. Androgenetic alopecia is defined as "vellus transformation" (van Scott and Ekel, 1958). For the mechanism of the action of minoxidil, no hormonal effect, but the direct effect on the follicular epithelium has been confirmed (Kulick, 1988; Olsen, 1989). It is reported that minoxidil induces a significant shortening of the telogen phase (Uno *et al*, 1985; Mori and Uno, 1990). It is thus possible to assume that procyanidin oligomers, which directly affect hair epithelial cell growth and stimulate anagen induction *in vivo,* will be effective for curing androgenetic alopecia.

We have already performed toxicologic studies (Takahashi *et al*, 1999) and a human pilot clinical study using procyanidin B-2, and confirmed that it is safe enough for topical use of procyanidin B-2 and effectiveness in curing androgenetic alopecia.[3] **We are now improving the industrial process for manufacturing procyanidin oligomers, and are planning a large-scale human clinical test.**

REFERENCES

Ariga T, Koshiyama I, Fukushima D: Antioxidative properties of procyanidins B-1 and B-3 from azuki beans in aqueous systems. *Agricultural and Biological Chemistry* 52:2717–2722, 1988

Barnard DL, Smee DF, Huffman JH, Meyerson LR, Sidwell RW: Antiherpesvirus activity and mode of action of SP-303, a novel plant flavonoid. *Chemotherapy* 39:203–211, 1993

Bernays EA, Driver GC, Bilgener M: Herbivores and plant tannins. *Advances Ecological Res* 19:263–302, 1989

Boeck C, Parker J, Shank J, Hordinsky M: Safety of long term therapy with 3% and 5% topical minoxidil in female androgenetic alopecia. In: van Neste D, Randall VA (eds). *Hair Research for the Next Millennium*. Amsterdam: Elsevier Science B.V., 1996, pp. 61–65

Boyce ST, Ham RG: Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J Invest Dermatol* 81:33S–40S, 1983

Brandon MJ, Foo LY, Porter LJ, Meredith P: Proanthocyanidins of barley and sorghum; composition as a function of maturity of barley ears. *Phytochemistry* 21:2953–2957, 1982

Buhl AE, Waldon DJ, Kawabe TT, Holland JM: Minoxidil stimulates mouse vibrissae follicles in organ culture. *J Invest Dermatol* 92:315–320, 1989

Buhl AE, Conrad SJ, Waldon DJ, Brunden MN: Potassium channel conductance as a control mechanism in hair follicles. *J Invest Dermatol* 101:148S–152S, 1993

Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB: Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 47:936–942, 1987

Cheng J-T, Hsu F-L, Chen H-F: Antihypertensive principles from the leaves of *Melastoma candidum*. *Planta Med* 59:405–407, 1993

Cotsarelis G, Sun T-T, Lavker RM: Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell* 61:1329–1337, 1990

Cui CB, Tezuka Y, Yamashita H, Kikuchi T, Nakano H, Tamaoki T, Park J-H: Constituents of a fern, *Davallia mariesii* Moore. V. Isolation and structures of davallin, a new tetrameric proanthocyanidin, and two new phenolic glycosides. *Chem Pharm Bull (Tokyo)* 41:1491–1497, 1993

Dallob AL, Sadick NS, Unger W, *et al*: The effect of finasteride, a 5α-reductase inhibitor, on scalp skin testosterone and dihydrotestosterone concentrations in patients with male pattern baldness. *J Clin Endocrinol Metab* 79:703–706, 1994

Danilenko DM, Ring BD, Pierce GF: Growth factors and cytokines in hair follicle development and cycling: recent insights from animal models and the potentials for clinical therapy. *Mol Med Today* 2:460–467, 1996

Dartenuc J-Y, Marache P, Choussat H: Résistance capillaire en gériatrie etude d'un microangioprotecteur = Endotélon. *Bordeaux Médical* 13:903–907, 1980

Eberhardt TL, Young RA: Conifer seed cone proanthocyanidin polymers: characterization by ^{13}C NMR spectroscopy and determination of antifungal activities. *J Agricultural Food Chemistry* 42:1704–1708, 1994

Facino RM, Carini M, Aldini G, Bombardelli E, Morazzoni P, Morelli R: Free radicals scavenging action and anti-enzyme activities of procyanidins from *Vitis vinifera*: a mechanism for their capillary protective action. *Arzneimittel-Forschung/ Drug Research* 44:592–601, 1994

Gali HU, Perchellet EM, Gao XM, Karchesy JJ, Perchellet JP: Comparison of the inhibitory effects of monomeric, dimeric, and trimeric procyanidins on the biochemical markers of skin tumor promotion in mouse epidermis *in vivo*. *Planta Med* 60:235–239, 1994

Gormley GJ: Finasteride: a clinical review. *Biomed Pharmacother* 49:319–324, 1995

Hattori M, Ogawa H: Biochemical analysis of hair growth from the aspects of aging and enzyme activities. *J Dermatol* 10:45–54, 1983

Hibberts NA, Howell AE, Randall VA: Balding hair follicle dermal papilla cells contain higher levels of androgen receptors than those from non-balding scalp. *J Endocrinol* 156:59–65, 1998

Hong C-Y, Wang C-P, Huang S-S, Hsu F-L: The inhibitory effect of tannins on lipid peroxidation of rat heart mitochondria. *J Pharm Pharmacol* 47:138–142, 1995

Jahoda C, Oliver RF: The growth of vibrissa dermal papilla cells *in vitro*. *Br J Dermatol* 105:623–627, 1981

Kashiwada Y, Nonaka G-I, Nishioka I, Ballas LM, Jiang JB, Janzen WP, Lee K-H: Tannins as selective inhibitors of protein kinase C. *Bioorganic Medicinal Chemistry Letters* 2:239–244, 1992

Kaufman KD, Olsen EA, Whiting D, *et al*: Finasteride in the treatment of men with androgenetic alopecia. *J. Am Acad Dermatol* 39: 578–589, 1998.

Kulick MI: Topical minoxidil: its use in treatment of male pattern baldness. *Ann Plast Surg* 21:273–275, 1988

Liviero L, Puglisi PP, Morazzoni P, Bombardelli E: Antimutagenic activity of procyanidins from *Vitis vinifera*. *Fitoterapia* LXV:203–209, 1994

McCormick MA, Forman MH, Manoguerra AS: Severe toxicity from ingestion of a topical minoxidil preparation. *Am J Emerg Med* 7:419–421, 1989

Messenger AG: The control of hair growth: an overview. *J Invest Dermatol* 101:4S–9S, 1993

Mori O, Uno H: The effect of topical minoxidil on hair follicular cycles of rats. *J Dermatol* 17:276–281, 1990

Morimoto S, Nonaka G-I, Nishioka I: Tannins and related compounds. VIII. Isolation and characterization of flavan-3-ol glucosides and procyanidin oligomers from cassia bark (*Cinnamomum cassia* Blume). *Chem Pharm Bull (Tokyo)* 34:633–642, 1986

Nickel JC, Fradet Y, Boake RC *et al*: Efficacy and safety of finasteride therapy for benign prostatic hyperplasia: results of a 2-year randomized controlled trial (the PROSPECT study). *Can Med Assoc J* 155:1251–1259, 1996

Nonaka G-I, Hsu F-L, Nishioka I: Structures of dimeric, trimeric, and tetrameric procyanidins from *Areca catechu* L. *J Chemical Soc Chemical Communications* 781–783, 1981

Nonaka G-I, Kawahara O, Nishioka I: Tannins and related compounds. VIII. A

[3]Takahashi T, Yokoo Y, Watanabe Y: A pilot clinical study of topical procyanidin B-2 with the aim of considering its application as a hair growing agent. (manuscript in preparation).

new type of proanthocyanidin, cinchonains IIa and IIb from *Cinchona succirubra*. *Chem Pharm Bull (Tokyo)* 30:4277–4282, 1982

Olsen EA: Treatment of androgenetic alopecia with topical minoxidil. *Resident and Staff Physician* 35:53–69, 1989

Paus R, Stenn KS, Link RE: The induction of anagen hair growth in telogen mouse skin by cyclosporin A administration. *Lab Invest* 60:365–369, 1989

Paus R, Heinzelmann T, Schultz K-D, Furkert J, Fechner K, Czarnetzki BM: Hair growth induction by substance P. *Lab Invest* 71:134–140, 1994

Polya GM, Foo LY: Inhibition of eukaryote signal-regulated protein kinases by plant-derived catechin-related compounds. *Phytochemistry* 35:1399–1405, 1994

Porter LJ. Flavans and proanthocyanidins. In: Harborne JB (ed.). *The Flavonoids: Advances in Research Since 1986*. London: Chapman & Hall 1994, pp. 23–55

Scalbert A: Antimicrobial properties of tannins. *Phytochemistry* 30:3875–3883, 1991

van Scott EJ, Ekel TM: Geometric relationships between the matrix of the hair bulb and its dermal papilla in normal and alopecic scalp. *J Invest Dermatol* 31:281–287, 1958

Takahashi T, Kamiya T, Yokoo Y: Proanthocyanidins from grape seeds promote proliferation of mouse hair follicle cells *in vitro* and convert hair cycle *in vivo*. *Acta Derm Venereol* 78:428–432, 1998

Takahashi T, Yokoo Y, Inoue T, Ishii A; Toxicological studies on procyanidin B-9 for external application as a hair growing agent. *Food Chem Toxicol* 1999, in press.

Tanigaki N, Ando H, Ito M, Hashimoto A, Kitano Y: Electron microscopic study of cultured cells from the murine hair tissues: cell growth and differentiation. *Arch Dermatol Res* 282:402–407, 1990

Tanigaki-Obana N, Ito M: Effects of cepharanthine and minoxidil on proliferation, differentiation and keratinization of cultured cells from the murine hair apparatus. *Arch Dermatol Res* 284:290–296, 1992

Thompson RS, Jacques D, Haslam E, Tanner RJN: Plant proanthocyanidins. Part I. Introduction; the isolation, structure and distribution in nature of plant procyanidins. *J Chem Soc*. Perkin Transactions 1: *Organic and Bio-organic Chemistry*, 1387–1399, 1972

Uno H, Cappas A, Schlagel C: Cyclic dynamics of hair follicles and the effect of minoxidil on the bald scalps of stumptailed macaques. *Am J Dermatopathol* 7:283–297, 1985

Vennat B, Gross D, Pourrat H, Pourrat A, Bastide P, Bastide J: Anti-ulcer activity of procyanidins: preparation of water-soluble procyanidin-cimetidine complexes. *Pharm Acta Helv* 64:316–320, 1989

Vennat B, Bos M-A, Pourrat A, Bastide P: Procyanidins from tormentil: fractionation and study of the anti-radical activity towards superoxide anion. *Biol Pharm Bull* 17:1613–1615, 1994

Wayne Z, Elementals LLC, Scottsdale AZ: Pycnogenol and skincare. *Drug and Cosmetic Industry* 158:44–50, 1996

Wilton L, Pearce G, Edet E, Freemantle S, Stephens MDB, Mann RD: The safety of finasteride used in benign prostatic hypertrophy: a non-interventional observational cohort study in 14772 patients. *Br J Urol* 78:379–384, 1996

Wysocki GP, Daley TD: Hypertrichosis in patients receiving cyclosporine therapy. *Clin Exp Dermatol* 12:191–196, 1987