

## Procyanidin Oligomers Counteract TGF- $\beta_1$ - and TGF- $\beta_2$ -Induced Apoptosis in Hair Epithelial Cells: An Insight into Their Mechanisms

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### Key Words

Apoptosis · MEK · TGF- $\beta$

### Abstract

Procyanidin oligomers are polyphenol compounds we have identified in apples and barley which have hair growth stimulant effects, and which are able to promote hair epithelial cell growth and induce anagen induction of the hair cycle in the in vivo murine model. For the purpose of examining the hair-growing mechanisms of procyanidin oligomers, we examined their relationship to the TGF- $\beta$  signal pathway, known to be a regulator of catagen induction, and the mitogen-activated protein kinase cascade linked to cell proliferation. Addition of TGF- $\beta_1$  or TGF- $\beta_2$  to hair epithelial cell cultures dose-dependently decreased cell growth and induced apoptosis; however, addition of procyanidin B-2 to the culture neutralized the growth-inhibiting effects of both TGF- $\beta_1$  and TGF- $\beta_2$  and protected the cells from apoptosis. The same effects were observed with procyanidin B-3. We confirmed that procyanidin B-2 upregulates the expression of MEK-1/2 in cultured murine hair epithelial cells. We speculate that the hair-growing activity of procyanidin oligomers is at least linked to their growth-promoting

effects on hair epithelial cells that follow MEK activation and their protective action on TGF- $\beta_1$ - or TGF- $\beta_2$ -induced apoptosis that is assumed to trigger catagen induction in the hair cycle.

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### Introduction

Several internal factors are known to regulate hair growth [1]: for example, IGF-1 [2] and HGF [3, 4] are known to positively regulate hair growth; TGF- $\beta$  [5], FGF-5 [6, 7], TNF- $\alpha$  [8], IL-1 $\alpha$  [8–10], and IL-1 $\beta$  [8, 11] are known to negatively regulate hair growth. On the other hand, external factors, such as plant-derived compounds [12], metabolites of microorganisms [13], and synthetic drugs [14], are also known to promote hair growth.

Proanthocyanidins are a species of phenolic compounds highly prevalent in plants; they take the form of polymers or oligomers built of flavan-3-ol units [15]. Proanthocyanidins have long been used in medications and cosmetics designated to protect the capillaries [16] and skin [17]. Numerous physiological and pharmacological effects are reported for proanthocyanidins, of which rad-

ical scavenging [18], antioxidant activity [19], antihypertensive activity [20], capillary protective action [21], and elastase-inhibiting action [22] are significant from the dermatological viewpoint.

We have recently found hair epithelial cell growth-promoting activity and the ability to induce the anagen phase *in vivo* in procyanidin oligomers purified from apples [23] and barley [24]. We identified the active compounds in apples as procyanidin B-1, procyanidin B-2 (fig. 1a), and procyanidin C-1, and the active compound in barley as procyanidin B-3 (fig. 1b).

This paper describes our investigation into the effects of procyanidin oligomers on murine hair epithelial cell growth, specifically their relationship to TGF- $\beta_1$ - and TGF- $\beta_2$ -induced cellular responses in hair epithelial cells, that is, apoptotic cell death [25], and the activation of the mitogen-activated protein kinase (MAPK) cascade.

In this report, we describe how procyanidin B-2 has the potential to upregulate MAPK/extracellular signal-regulated kinase kinase (MEK) in hair epithelial cells, and how procyanidin oligomers have the potential to protect hair epithelial cells from TGF- $\beta_1$ - and TGF- $\beta_2$ -induced apoptotic cell death that is assumed to trigger catagen induction [5, 26–28] in the hair cycle.

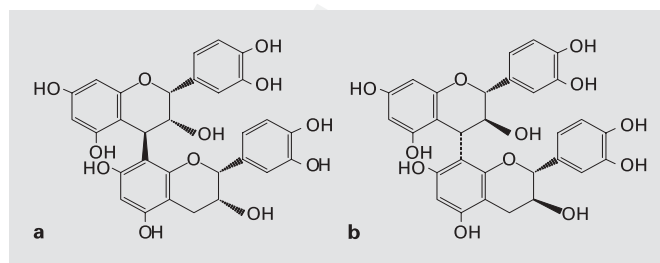
## Materials and Methods

### Materials

Procyanidin B-2 [(-)-epicatechin-(4 $\beta$ →8)-(-)-epicatechin] (fig. 1a) was obtained from apple juice (*Malus pumila* Miller var. *domestica* Schneider, Fuji variety, commercial juice) according to the method described in a previous report [23]. The product was identified using mass spectrometry,  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR [29, 30]. Procyanidin B-3 [(+)-catechin-(4 $\alpha$ →8)-(+)-catechin] (fig. 1b) was obtained from seed husks of barley (*Hordeum vulgare* L. var. *distichon* Alefeld) according to the method described in a previous report [24]. The product was identified using mass spectrometry,  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR [29, 31, 32]. TGF- $\beta_1$  and TGF- $\beta_2$  (human recombinant) were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Affinity-purified polyclonal antibodies against MEK-1/2 (rabbit antihuman) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). The secondary antibody used was horseradish peroxidase-conjugated goat antirabbit immunoglobulin purchased from DAKO (Glostrup, Denmark).

### Isolation and Culturing of Hair Epithelial Cells

Murine hair epithelial cells were isolated from neonatal C3H/HeNCrj mice (Charles River Japan, Inc., Kanagawa, Japan) according to the method described in another report [33]. Before starting our experiments, we confirmed, by the keratin pattern expressed in the cultured cells (data not shown), that the isolated cells were hair epithelial cells [34]. The purity of the isolated hair epithelial cells is also confirmed in electron microscopic studies [35, 36]. The dorsal skin was peeled from 4-day-old C3H/HeNCrj mice,



**Fig. 1.** Structure of procyanidin B-2 [(-)-epicatechin-(4 $\beta$ →8)-(-)-epicatechin] (a) and procyanidin B-3 [(+)-catechin-(4 $\alpha$ →8)-(+)-catechin] (b).

cut into about 5-mm widths, then dipped into Eagle's minimum essential medium containing 750 IU/ml of dispase (from *Bacillus polymyxa*; Godo Shusei Co., Tokyo, Japan), 60 mg/l of kanamycin, and 10% (v/v) 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's medium containing 0.25% (w/v) collagenase (from *Streptomyces parvulus*; Nitta Gelatin Co., Osaka, Japan), 50,000 U/l of penicillin, 50 mg/l of streptomycin, 0.5% (w/v) bovine serum albumin and 20% (v/v) FCS at 37°C for 1 h, stirring occasionally. This dermis suspension was filtered through a 212- $\mu\text{m}$  nylon mesh, and the filtrate was centrifuged at 1,400 rpm for 7 min. The pellet was resuspended in Dulbecco's phosphate-buffered calcium- and magnesium-free saline containing 50,000 U/l of penicillin and 50 mg/l of 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% (w/v) EDTA-0.25% (w/v) trypsin in Hanks' balanced calcium- and magnesium-free salt solution (Life Technologies, Inc., Md., USA) at 37°C for 5 min. The hair follicle cells were suspended in Dulbecco's modified Eagle's medium supplemented with 50,000 U/l of penicillin, 50 mg/l of streptomycin, and 10% (v/v) FCS at a density of  $3 \times 10^5$  cells/ml after filtration via a 212- $\mu\text{m}$  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 24 h incubation, the medium was exchanged with MCDB 153 (Sigma) containing 5 mg/l of bovine insulin, 5  $\mu\text{g}$ /l of mouse EGF, 40 mg/l of bovine pituitary extract, 10 mg/l of human transferrin, 0.4 mg/l of hydrocortisone, 0.63  $\mu\text{g}$ /l of progesterone, 14 mg/l of *o*-phosphorylethanolamine, 6.1 mg/l of ethanolamine, 50,000 U/l of penicillin, and 50 mg/l of streptomycin. It was then further incubated in a humidified atmosphere containing 5% CO $_2$  at 37°C. During incubation, the medium was removed and replaced with fresh medium every other day. After 5 days, the degree of cell growth was determined by MTT assay and apoptotic cell death was detected using ELISA.

### 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

[37]. To summarize, MTT reagent was dissolved in Dulbecco's calcium- and magnesium-free PBS at a concentration of 5 mg/ml, filtered through a 0.45- $\mu$ m membrane filter (cellulose acetate, DIS-MIC-13cp, Advantec, Tokyo, Japan), and 10% (v/v) were added to the culture medium. The culture plate was further incubated in a humidified atmosphere containing 5% CO<sub>2</sub> 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<sup>2</sup> well), and the absorbance was measured at 570 nm relative to 640 nm. As controls, we placed cell-free blank, in which test-agent-containing medium was pipetted, in the culture plates. Growth-promoting activities relative to controls (= 100%) were calculated. Results are expressed as mean  $\pm$  SD (n = 6) carried out with primary cultures.

#### *Detection of Apoptotic Cell Death in Cultured Hair Epithelial Cells Using ELISA*

Apoptotic cell death was quantitatively measured by detecting histone-associated DNA fragments using the sandwich ELISA system (Cell Death Detection ELISA<sup>PLUS</sup>, Boehringer Mannheim). Both the cell lysate and the culture medium supernatant were subjected to analysis. The level of histone-associated DNA fragments relative to controls (= 100%) was calculated. Results are expressed as mean  $\pm$  SD (n = 3) carried out with primary cultures.

#### *Immunoblot Analysis (Western Blotting)*

The primary cultured murine hair epithelial cell pellet was sonicated in five 10-second bursts in buffer A [20 mM Tris(hydroxymethyl)aminomethane(Tris)-HCl (pH 7.5), 2 mM EDTA, 10 mM EGTA, 0.25 M sucrose, 2 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin, and 10 mM 2-mercaptoethanol; final concentrations] and centrifuged at 100,000 g for 60 min (4°C). The supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis according to the method described in another report [38] with minor modifications. For the detection of MEK-1/2, we used a polyclonal antibody against MEK-1/2 (rabbit antihuman, Santa Cruz Biotechnology) as the primary antibody, and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (DAKO) as the secondary antibody.

#### *Statistical Analysis*

All data are expressed as mean  $\pm$  standard deviation. The data of the cell proliferation level and apoptotic cell death shown in figure 2 were analyzed by Dunnett's multiple comparison test after the one-way analysis of variance. MEK-1/2 expression in figure 3 was analyzed by paired t test. Statistical significance was set at p < 0.05.

## **Results**

### *Addition of TGF- $\beta$ <sub>1</sub> or TGF- $\beta$ <sub>2</sub> Dose-Dependently Decreases Hair Epithelial Cell Growth and Induces Apoptosis in Hair Epithelial Cells; Addition of Procyanidin B-2 or Procyanidin B-3 to the Culture Counteracts the Inhibitory Effects of Both TGF- $\beta$ <sub>1</sub> and TGF- $\beta$ <sub>2</sub>*

Of the many hair-growth-regulating factors known, TGF- $\beta$  has of late been a focus of increasing research in-

terest, since it is a potent negative hair-growing factor that induces the catagen phase of the hair cycle [5, 26–28]. We examined the effects of TGF- $\beta$ <sub>1</sub> and TGF- $\beta$ <sub>2</sub> on murine hair epithelial cell growth and subsequently observed that addition of TGF- $\beta$ <sub>1</sub> to the culture medium dose-dependently inhibits murine hair epithelial cell growth and induces apoptotic cell death (fig. 2a, c). The same effects were observed with TGF- $\beta$ <sub>2</sub> (fig. 2b, d). Addition of procyanidin B-2 to the medium containing TGF- $\beta$ <sub>1</sub> almost entirely counteracted the growth-inhibitory effects of TGF- $\beta$ <sub>1</sub>, and protected the cells from apoptosis (fig. 2a). The same results were obtained with a combination of procyanidin B-2 and TGF- $\beta$ <sub>2</sub> (fig. 2b). Procyanidin B-3 similarly counteracted the inhibitory effects of both TGF- $\beta$ <sub>1</sub> and TGF- $\beta$ <sub>2</sub>, and protected the cells from apoptosis (fig. 2c, d). Micrographs of murine hair epithelial cells cultured in the presence of TGF- $\beta$ <sub>1</sub>, TGF- $\beta$ <sub>2</sub>, or procyanidin B-2, and in the combinations of TGF- $\beta$ <sub>1</sub> and procyanidin B-2, and TGF- $\beta$ <sub>2</sub> and procyanidin B-2, are shown in figure 4.

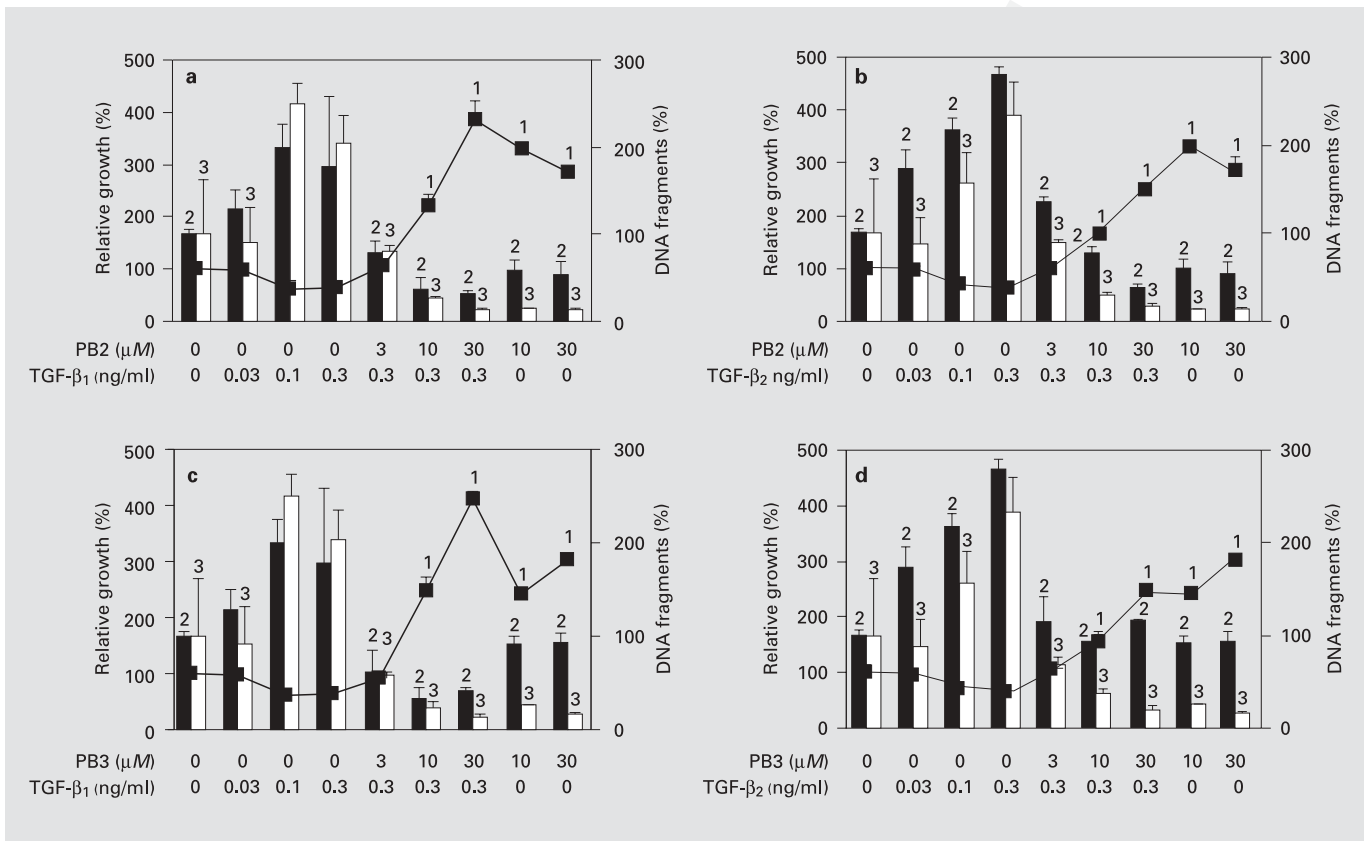
### *Procyanidin B-2 Upregulates the Level of MEK-1/2 in Cultured Murine Hair Epithelial Cells*

Using Western blotting, we examined the effects of procyanidin B-2 on the expression of MEK-1/2 in cultured murine hair epithelial cells. The hair epithelial cells were incubated in media containing 10  $\mu$ M (= 5.79  $\mu$ g/ml) of procyanidin B-2 for the final 48 h of the 5-day culture period. We observed increases in the expression of MEK-1/2 in hair epithelial cells cultured in media containing 10  $\mu$ M (= 5.79  $\mu$ g/ml) of procyanidin B-2 of 434  $\pm$  47% (mean  $\pm$  SD; paired t test, p < 0.001) relative to the controls (= 100%) (fig. 3).

## **Discussion**

Several internal factors are known to regulate hair growth [1]: for example, IGF-1, HGF, KGF and VEGF are known to positively regulate hair growth; TGF- $\beta$ , FGF-5, TNF- $\alpha$ , IL-1 $\alpha$ , and IL-1 $\beta$  are known to negatively regulate hair growth.

Of these growth factors and cytokines reported to act on hair follicles, TGF- $\beta$ <sub>1</sub> has been considered to be a potent triggering factor of catagen induction in the murine hair cycle [5, 26]. TGF- $\beta$ <sub>2</sub> has been hypothesized to be a catagen-inducing factor in humans [27, 28]. TGF- $\beta$ <sub>1</sub> is also known as an apoptosis-inducing factor [25] in many types of cells [39]. As for its effects on skin keratinocytes, Shipley et al. [40] reported that TGF- $\beta$  causes

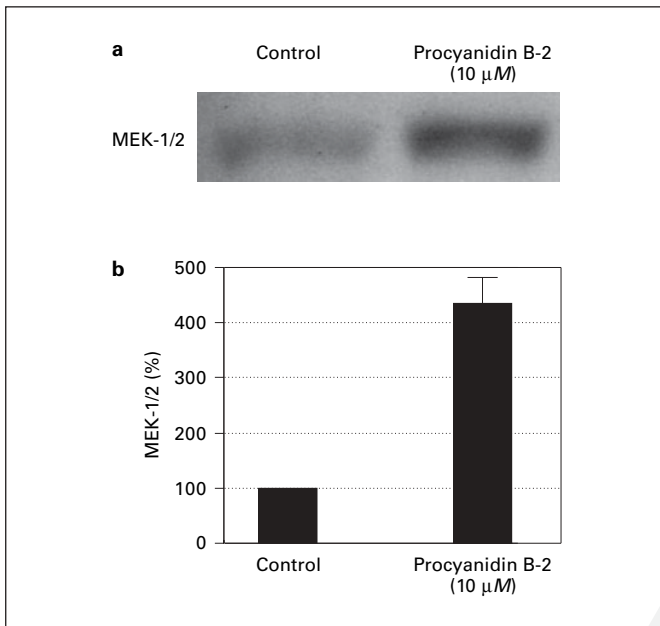


**Fig. 2.** Levels of hair epithelial cell growth and apoptotic cell death caused by TGF-β<sub>1</sub> or TGF-β<sub>2</sub>, and the effects of procyanidin B-2 and procyanidin B-3. The growth-promoting activities (■) on hair epithelial cells were measured by MTT assay. The values relative to controls (= 100%) are shown. Apoptotic cell death was quantitatively measured by detecting mono- and oligonucleosomes both in the cell lysate (solid bar) and the culture medium supernatant (open bar) by ELISA assay. DNA fragments in the cell lysate and the culture medium supernatant relative to controls (= 100%) are shown. TGF-β and procyanidin oligomers were added to the culture during the last 5 days. For the control, a medium without TGF-β (TGF-β<sub>1</sub> or TGF-β<sub>2</sub>) or procyanidin oligomers (procyanidin B-2 or procyanidin B-3) was used. Results are expressed as mean ± SD carried out with primary cultures prepared from 50 neonatal mice. **a** Effects of procyanidin B-2 (PB2) on TGF-β<sub>1</sub>-induced apoptosis. **b** Effects of procyanidin B-2 (PB2) on TGF-β<sub>2</sub>-induced apoptosis.

**c** Effects of procyanidin B-3 (PB3) on TGF-β<sub>1</sub>-induced apoptosis. **d** Effects of procyanidin B-3 (PB3) on TGF-β<sub>2</sub>-induced apoptosis. Both TGF-β<sub>1</sub> and TGF-β<sub>2</sub> dose-dependently repress hair epithelial cell growth and cause apoptotic cell death. Addition of procyanidin B-2 counteracts the growth-inhibitory effect of both TGF-β<sub>1</sub> and TGF-β<sub>2</sub> and protects the cells from apoptosis. The same results are obtained with procyanidin B-3. The cell proliferation level of each group was compared to 0 ng/ml TGF-β<sub>1</sub> or TGF-β<sub>2</sub> plus 0 μM PB2 group using Dunnett's multiple comparison method (<sup>1</sup>p < 0.05). The level of histone-associated DNA fragments in the cell lysate of each group was compared to 0.3 ng/ml TGF-β<sub>1</sub> or TGF-β<sub>2</sub> plus 0 μM PB2 group using Dunnett's multiple comparison method (<sup>2</sup>p < 0.05). The level of histone-associated DNA fragments in the supernatant of each group was compared to 0.3 ng/ml TGF-β<sub>1</sub> or TGF-β<sub>2</sub> plus 0 μM PB2 group using Dunnett's multiple comparison method (<sup>3</sup>p < 0.05).

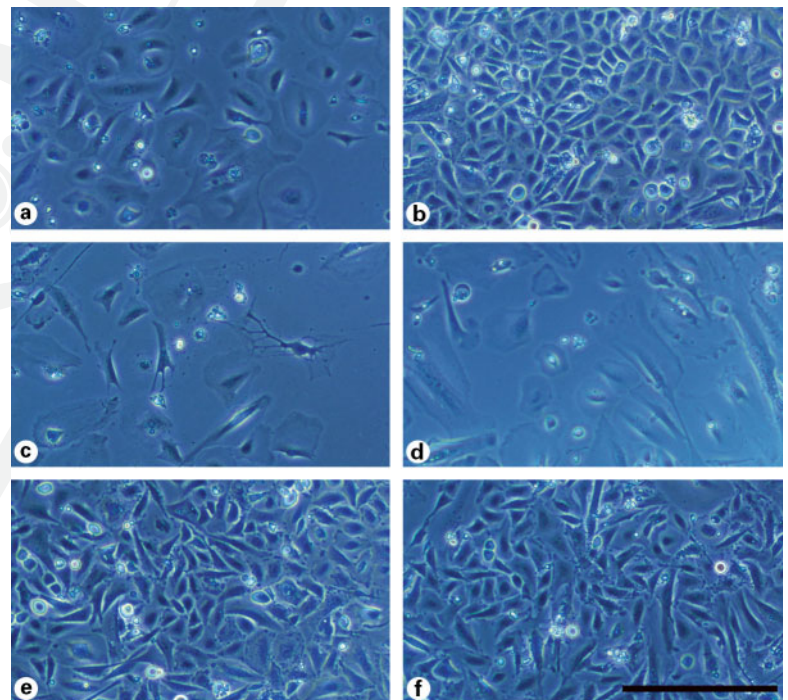
G<sub>1</sub> arrest of the cell cycle in human keratinocytes and Benassi et al. [41] also reported that TGF-β<sub>1</sub> causes apoptosis in cultured human keratinocytes. We examined the effect of TGF-β<sub>1</sub> on murine hair epithelial cell growth, and observed that the addition of TGF-β<sub>1</sub> to the culture medium dose-dependently inhibits the growth of murine hair epithelial cells and induces apoptotic cell death. Addition of procyanidin oligomers to medium

containing TGF-β<sub>1</sub> completely counteracted TGF-β<sub>1</sub>-induced apoptotic cell death. The same effects were observed with TGF-β<sub>2</sub>. From these results, we conclude that the hair-growing mechanisms of procyanidin oligomers are at least attributable to inhibition of TGF-β<sub>1</sub>- or TGF-β<sub>2</sub>-induced apoptotic cell death in hair epithelial cells.



**Fig. 3.** Western blotting analytical results for MEK-1/2 extracted from cultured murine hair epithelial cells (**a**). Procyanidin B-2 upregulates the level of MEK-1/2 in cultured murine hair epithelial cells. Procyanidin B-2, to obtain a concentration of 10  $\mu M$  (= 5.79  $\mu g/ml$ ), was added to the culture medium during the final 48 h of the 5-day culture period. Quantitative analysis was performed by densitometry (**b**). The level of MEK-1/2 in the control is expressed as 100.

One mechanism for the growth-promoting effect of procyanidin oligomers on hair epithelial cells appears to be the activation of the MAPK (= extracellular signal-regulated protein kinase) cascade, an important pathway that occurs during cell proliferation. As possible mechanisms of the inhibitory effect of procyanidin oligomers on TGF- $\beta$ -induced apoptosis in hair epithelial cells, the following is speculated. One possibility is an antioxidant-related mechanism. It is known that TGF- $\beta$ -induced apoptosis is mitochondria-dependent [42] and mediated by oxidative stress, i.e., caused by hydrogen peroxide [43]. The results of experiments using HIT cells, a hamster pancreatic  $\beta$ -cell line, suggest that TGF- $\beta_1$  enhances apoptosis through the suppression of antioxidative enzymes, catalase and glutathione peroxidase [44]. It is known that procyanidin oligomers have intensive antioxidative activity [19]. It can thus be speculated that procyanidin oligomers exert an inhibitory effect on TGF- $\beta$ -induced apoptosis through blocking oxidative stress caused by TGF- $\beta$ . On the other hand, an MAPKK (= MEK), Bcl- $x_L$  route can be speculated. It has been reported that activation of MAPKK (= MEK) upregulates the expression of Bcl- $x_L$  [45], an antiapoptotic factor [46]. We confirmed that procyanidin B-2 upregulates MEK in hair epithelial cells (fig. 3). These items of information, when taken together, suggest that the hair-growing mech-



**Fig. 4.** Micrographs of hair epithelial cells cultured in the presence of TGF- $\beta_1$ , TGF- $\beta_2$ , or procyanidin B-2, and cultured in the presence of both procyanidin B-2 and TGF- $\beta$  (TGF- $\beta_1$  or TGF- $\beta_2$ ). Hair epithelial cells cultured for 5 days are shown. **a** Control of cultured murine hair epithelial cells. **b** Murine hair epithelial cells cultured in the presence of 30  $\mu M$  (= 17.4  $\mu g/ml$ ) procyanidin B-2. **c** Murine hair epithelial cells cultured in the presence of 0.3 ng/ml of TGF- $\beta_1$ . **d** Murine hair epithelial cells cultured in the presence of 0.3 ng/ml of TGF- $\beta_2$ . **e** Murine hair epithelial cells cultured in the presence of both 0.3 ng/ml of TGF- $\beta_1$  and 30  $\mu M$  (= 17.4  $\mu g/ml$ ) procyanidin B-2. **f** Murine hair epithelial cells cultured in the presence of both 0.3 ng/ml of TGF- $\beta_2$  and 30  $\mu M$  procyanidin B-2. Scale bars: 100  $\mu m$ .

anism of procyanidin oligomers is at least partially based on its inhibitory effects on TGF- $\beta$ -induced apoptotic cell death in hair epithelial cells through both MEK activation and their antioxidative action.

We speculate that the hair-growing activity of procyanidin oligomers is at least linked to their growth-promot-

ing effect on hair epithelial cells and their protective action on TGF- $\beta_1$ - or TGF- $\beta_2$ -induced apoptotic cell death that is assumed to trigger catagen induction [5, 26] in the hair cycle. Both mechanisms are speculated to be linked to activation of the MAPK signaling pathway.

## References

- 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* 1996;2:460–467.
- Philpott MP, Sanders DA, Kealey T: Effects of insulin and insulin-like growth factors on cultured human hair follicles: IGF-1 at physiologic concentrations is an important regulator of hair follicle growth in vitro. *J Invest Dermatol* 1994;102:857–861.
- Jindo T, Tsuboi R, Imai R, Takamori K, Rubin JS, Ogawa H: The effect of hepatocyte growth factor/scatter factor on human hair follicle growth. *J Dermatol Sci* 1995;10:229–232.
- Jindo T, Tsuboi R, Takamori K, Ogawa H: Local injection of hepatocyte growth factor/scatter factor (HGF/SF) alters cyclic growth of murine hair follicles. *J Invest Dermatol* 1998;110:338–342.
- Seiberg M, Marthinuss J, Stenn KS: Changes in expression of apoptosis-associated genes in skin mark early catagen. *J Invest Dermatol* 1995;104:78–82.
- Hébert JM, Rosenquist T, Götz J, Martin GR: FGF5 as a regulator of the hair growth cycle: evidence from targeted and spontaneous mutations. *Cell* 1994;78:1017–1025.
- Pethö-Schramm A, Müller H-J, Paus R: FGF5 and the murine hair cycle. *Arch Dermatol Res* 1996;288:264–266.
- Philpott MP, Sanders DA, Bowen J, Kealey T: Effects of interleukins, colony-stimulating factor and tumour necrosis factor on human hair follicle growth in vitro: a possible role for interleukin-1 and tumour necrosis factor- $\alpha$  in alopecia areata. *Br J Dermatol* 1996;135:942–948.
- Harmon CS, Nevins TD: IL-1 $\alpha$  inhibits human hair follicle growth and hair fiber production in whole-organ cultures. *Lymphokine Cytokine Res* 1993;12:197–203.
- Mahé YF, Buan B, Billoni N, Loussouarn G, Michelet J-F, Gautier B, Bernard BA: Pro-inflammatory cytokine cascade in human plucked hair. *Skin Pharmacol* 1996;9:366–375.
- Xiong Y, Harmon CS: Interleukin-1 $\beta$  is differentially expressed by human dermal papilla cells in response to PKC activation and is a potent inhibitor of human hair follicle growth in organ culture. *J Interferon Cytokine Res* 1997;17:151–157.
- 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* 1992;284:290–296.
- Taylor M, Ashcroft ATT, Messenger AG: Cyclosporin A prolongs human hair growth in vitro. *J Invest Dermatol* 1993;100:237–239.
- Buhl AE, Waldon DJ, Kawabe TT, Holland JM: Minoxidil stimulates mouse vibrissae follicles in organ culture. *J Invest Dermatol* 1989;92:315–320.
- Porter LJ: Flavans and proanthocyanidins; in Harborne JB (ed): *The Flavonoids: Advances in Research since 1986*. London, Chapman and Hall, 1994, pp 23–55.
- Dartenuc J-Y, Marache P, Choussat H: Résistance capillaire en gériatrie: étude d'un microangioprotecteur – Endotélon. *Bord Méd* 1980;13:903–907.
- Wayne Z: Pycnogenol and skin care. *Drug Cosmet Ind* 1996;158:44–50.
- 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* 1994;17:1613–1615.
- 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* 1995;47:138–142.
- Cheng J-T, Hsu F-L, Chen H-F: Antihypertensive principles from the leaves of *Melastoma candidum*. *Planta Med* 1993;59:405–407.
- 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. *Arzneimittelforschung* 1994;44:592–601.
- Tixier JM, Godeau G, Robert AM, Hornebeck W: Evidence by in vivo and in vitro studies that binding of pycnogenols to elastin affects its rate of degradation by elastases. *Biochem Pharmacol* 1984;33:3933–3939.
- Takahashi T, Kamiya T, Hasegawa A, Yokoo Y: Procyanidin oligomers selectively and intensively promote proliferation of mouse hair epithelial cells in vitro and activate hair follicle growth in vivo. *J Invest Dermatol* 1999;112:310–316.
- Kamimura A, Takahashi T: Procyanidin B-3, isolated from barley and identified as a hair-growth stimulant, has the potential to counteract inhibitory regulation by TGF- $\beta_1$ . *Exp Dermatol* 2002;11:532–541.
- Schuster N, Kriegelstein K: Mechanisms of TGF- $\beta$ -mediated apoptosis. *Cell Tissue Res* 2002;307:1–14.
- Foitzik K, Lindner G, Mueller-Roeber S, Maurer M, Botchkareva N, Botchkarev V, Handjiski B, Metz M, Hibino T, Soma T, Dotto GP, Paus R: Control of murine hair follicle regression (catagen) by TGF- $\beta_1$  in vivo. *FASEB J* 2000;14:752–760.
- Soma T, Tsuj, Y, Hibino T: Involvement of transforming growth factor- $\beta_2$  in catagen induction during the human hair cycle. *J Invest Dermatol* 2002;118:993–997.
- Hibino T, Nishiyama T: Role of TGF- $\beta_2$  in the human hair cycle. *J Dermatol Sci* 2004;35:9–18.
- Thompson RS, Jacques D, Haslam E, Tanner RJN: Plant proanthocyanidins. 1. Introduction: the isolation, structure and distribution in nature of plant procyanidins. *J Chem Soc Perkin Trans I* 1972;1387–1399.
- Morimoto S, Nonaka G, Nishioka I: Tannins and related compounds. 38. Isolation and characterization of flavan-3-ol glucosides and procyanidin oligomers from cassia bark (*Cinnamomum cassia* Blume). *Chem Pharm Bull* 1986;34:633–642.
- Brandon MJ, Foo LY, Porter LJ, Meredith P: Proanthocyanidins of barley and sorghum: composition as a function of maturity of barley ears. *Phytochemistry* 1982;21:2953–2957.
- Newman RH, Porter LJ, Foo LY, Johns SR, Willing RI: High-resolution  $^{13}\text{C}$  NMR studies of proanthocyanidin polymers (condensed tannins). *Magn Reson Chem* 1987;25:118–124.
- Takahashi T: Biological actions of oligomeric procyanidins: proliferation of epithelial cells and hair follicle growth; in Packer L (ed): *Flavonoids and Other Polyphenols*. Methods Enzymol. San Diego, Academic Press, 2001, vol 335, pp 361–368.
- Bertolino AP, Gibbs PEM, Freedberg IM: In vitro biosynthesis of mouse hair keratins under the direction of follicular RNA. *J Invest Dermatol* 1982;79:173–177.

- 35 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* 1990; 282:402–407.
- 36 Tanigaki N, Ito M, Ando H, Kitano Y, Hashimoto A, Masamoto Y: Calcium regulation of growth, differentiation and keratinization of cultured cells from murine hair and hair follicles: an ultrastructural study. *Acta Med Biol* 1990;38:187–192.
- 37 Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB: Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 1987;47:936–942.
- 38 Takahashi T, Kamimura A: Cyclosporin A promotes hair epithelial cell proliferation and modulates protein kinase C expression and translocation in hair epithelial cells. *J Invest Dermatol* 2001;117:605–611.
- 39 Moustakas A, Pardali K, Gaal A, Heldin CH: Mechanisms of TGF- $\beta$  signaling in regulation of cell growth and differentiation. *Immunol Lett* 2002;82:85–91.
- 40 Shipley GD, Pittelkow MR, Wille JJ Jr, Scott RE, Moses HL: Reversible inhibition of normal human prokeratinocyte proliferation by type  $\beta$  transforming growth factor-growth inhibitor in serum-free medium. *Cancer Res* 1986;46:2068–2071.
- 41 Benassi L, Ottani D, Fantini F, Marconi A, Chiodino C, Giannetti A, Pincelli C: 1,25-Dihydroxyvitamin D<sub>3</sub>, transforming growth factor  $\beta$ <sub>1</sub>, calcium, and ultraviolet B radiation induce apoptosis in cultured human keratinocytes. *J Invest Dermatol* 1997;109:276–282.
- 42 Herrera B, Álvarez AM, Sánchez A, Fernández M, Roncero C, Benito M, Fabregat I: Reactive oxygen species (ROS) mediates the mitochondrial-dependent apoptosis induced by transforming growth factor  $\beta$  in fetal hepatocytes. *FASEB J* 2001;15:741–751.
- 43 Lee SD, Lee BD, Han JM, Kim JH, Kim Y, Suh P-G, Ryu SH: Phospholipase D<sub>2</sub> activity suppresses hydrogen peroxide-induced apoptosis in PC12 cells. *J Neurochem* 2000;75:1053–1059.
- 44 Islam KN, Kayanoki Y, Kaneto H, Suzuki K, Asahi M, Fujii J, Taniguchi N: TGF- $\beta$ <sub>1</sub> triggers oxidative modifications and enhances apoptosis in HIT cells through accumulation of reactive oxygen species by suppression of catalase and glutathione peroxidase. *Free Radic Biol Med* 1997;22:1007–1017.
- 45 Jost M, Huggett TM, Kari C, Boise LH, Roedel U: Epidermal growth factor receptor-dependent control of keratinocyte survival and Bcl-x<sub>L</sub> expression through a MEK-dependent pathway. *J Biol Chem* 2001;276:6320–6326.
- 46 Stoll SW, Benedict M, Mitra R, Hiniker A, Elder JT, Nunez G: EGF receptor signaling inhibits keratinocyte apoptosis: evidence for mediation by Bcl-x<sub>L</sub>. *Oncogene* 1998;16:1493–1499.