LABORATORIES

Product Information Booklet & Associated Medical Studies

Spectral.DNC-L®

TOPICAL TREATMENT FOR

ADVANCED STAGES OF BALDNESS

RESULTS

Spectral.DNC-L® delivers most comprehensive formula to stop hair loss and begin regrowth.

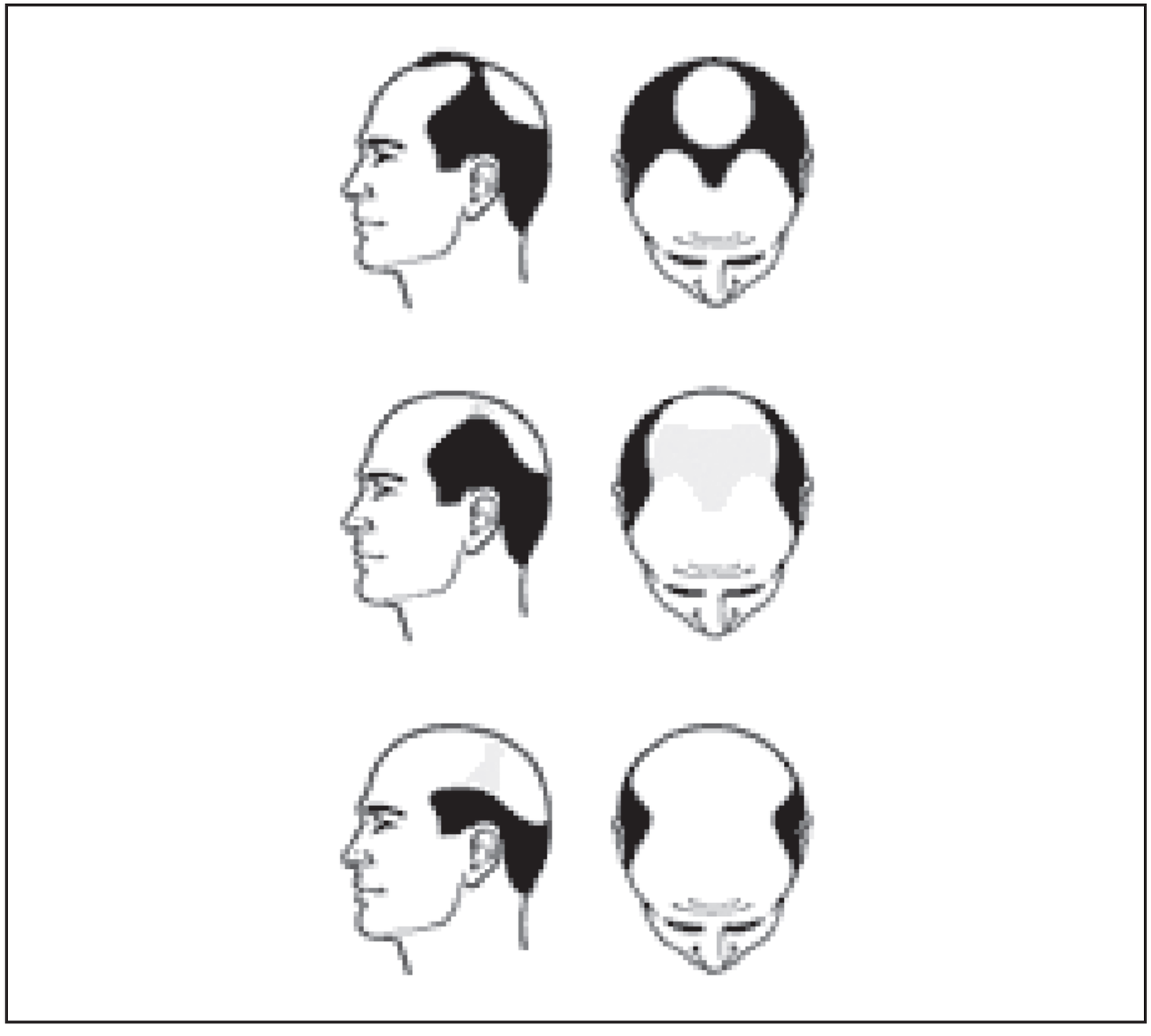
FOR MEN WITH ADVANCED androgenic alopecia (baldness of 4 or greater on the Norwood scale), Spectral.DNC-L delivers the most powerful, effective, and aggressive topical treatment to retain and

regrow hair, because Spectral.DNC-L brings together a broad spectrum of hair-growth compounds proven effective through clinical trials.

In a cream base for easy application without dripping, the potent formula employs high-grade 5% minoxidil, the leading growth agent, plus three advanced phytosterol 5-alpha-reductase inhibitors to suppress the dihydrotestosterone that triggers male pattern baldness, powerful antioxidants to detoxify free radicals, emerging procyanidins to

support epithelial and mesenchymal cell interaction, pioneering arginine to stimulate nitric oxide for follicle activity, natural botanicals to combat fungal colonization and reduce sebum, and retinol to calm inflamed tissues. The formula also contains Aminexil® to strengthen roots, and vitamins and minerals for nutrition.

This formula is just the beginning. Proprietary nanosome encapsulation delivers ingredients deeper into the scalp skin, where they can truly per-



Norwood scale from 4 (top) to 7 (bottom): Spectral.DNC-L is indicated for men with pattern baldness of 4 or greater.

form, wrapped in micro-spheres of organic nanosome, each 200 times smaller than a human skin cell.

This coordinated attack on baldness grows hair longer, thicker, and faster than other products, so you can live the lifestyle you really want.

INDICATIONS

Advanced cases of androgenic alopecia call for polyatomic Spectral.DNC-L®.

ALL TREATMENTS work better in the early stages of baldness, since delicate hair follicles tend to atrophy over the years. However, the polyatomic Spectral.DNC-L is indicated for men with advanced androgenic alopecia — male pattern baldness of 4 or greater on the Norwood scale — because it combines several complementary hair-growth compounds that perform through separate pathways.

These proven ingredients include minoxidil,

procyanidin B-2, arginine, adenosine, three 5-alpha-reductase inhibitors, and an army of compounds to enhance their performance. Together, the components of this breakthrough product treat follicular dysfunction more aggressively and effectively. Many patients who perceive little or no hair retention, thickening, or anagen-phase growth from minoxidil alone, do perceive dramatic improvement after three months of using Spectral.DNC-L.

The premium formulation also uses less alcohol, propolene glycol, and other fillers. Instead, Spectral.DNC-L is rich with extracts to soothe, moisturize, and condition skin, not inflame it further.

If genetic factors cause your hair loss, then treatment must continue to retain your new hair. Otherwise, follicles would regress in two or three

months. While Spectral.DNC-L does control androgenic alopecia, as yet no formula cures it. Once new hair is achieved, patients can switch to Spectral.DNC® for maintenance.

TECHNOLOGY

Welcome to the high-performance high-tech world of DS Laboratories.

DERMATOLOGISTS, cosmetologists, and users herald the treatments of DS Laboratories as best in each category because the company only exists to develop high-performance formulas. Spectral.DNC-L® — the state of the art for preserv-

ing and growing hair — results from cutting-edge research. We took a blank-slate approach to understanding hair loss, and a global approach to treating it aggressively.

Among many important innovations, Spectral .DNC-L delivers compounds of unparalleled purity through nanosome encapsulation. With this proprietary technology, micro-spheres of organic liposome, each 200 times smaller than a human skin cell, deposit their active ingredients deep below the surface of the skin, where they dissolve over 12 hours for maximum performance.

DS Laboratories obtains these precious compounds naturally through gentle mechanical compression, not by harsh chemical extraction. This high-purity method preserves molecular integrity and bioactivity. It is a costly process that

results in a lower yield. Still, the outstanding effectiveness of DS Laboratories' treatments does make the benefit worth the investment.

MECHANISMS OF ACTION

Minoxidil 5% is the best-known treatment to save and regrow hair.

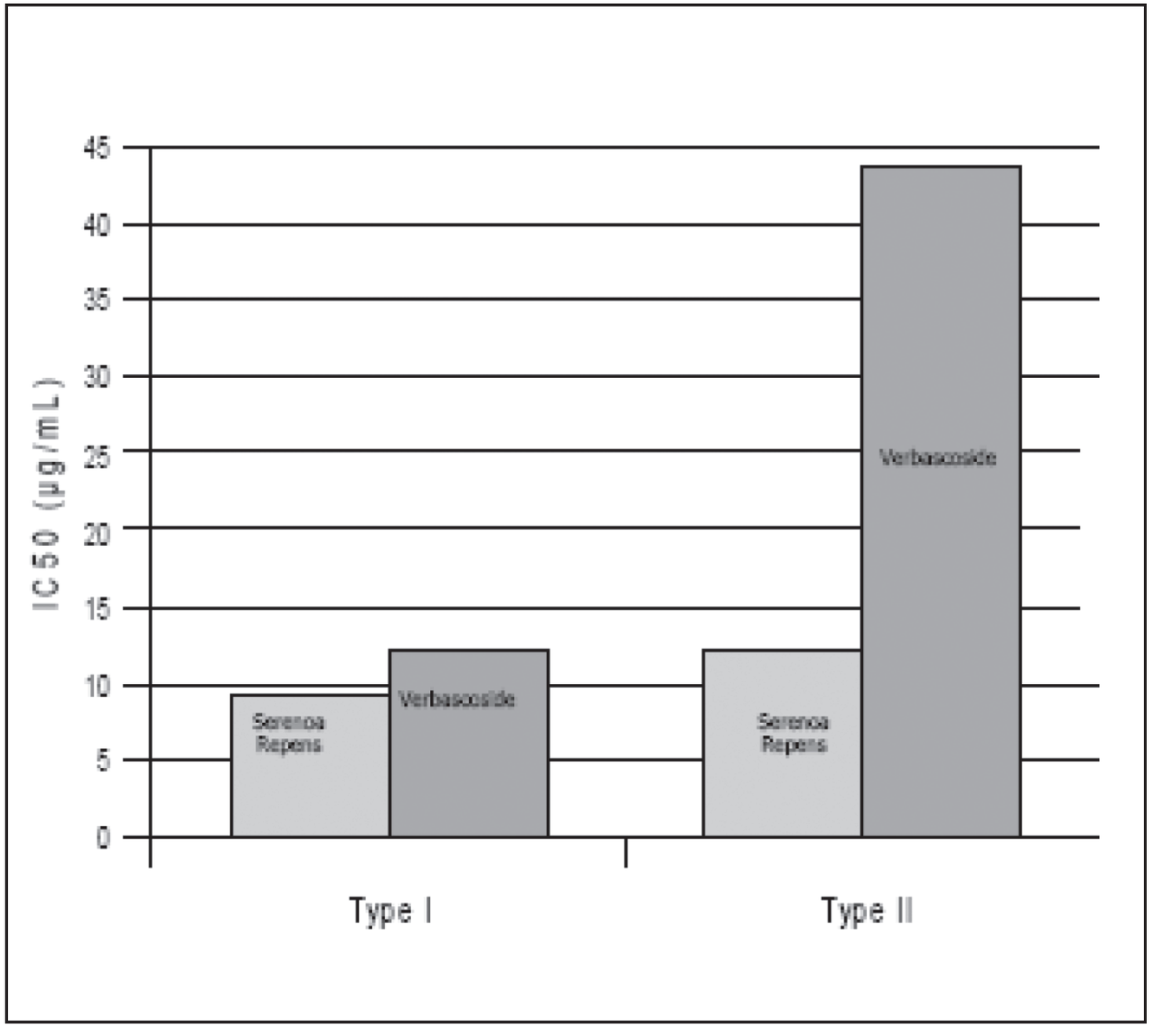
SPECTRAL.DNC-L® starts with 5% minoxidil because it revolutionized the treatment of alopecia, becoming the first drug approved by the US Food and Drug Administration to treat male pattern baldness. It remains the most widely used treatment today.

Before Rogaine®, the first topical formula with minoxidil, the vasodilator was used orally to treat high blood pressure. But the researchers discovered an interesting side effect: substantial hair growth on test subjects.

Subsequently, hundreds of clinical trials using thousands of volunteers have proven that minoxidil does regrow hair on the vertex of the scalp, although medical science does not yet fully understand the exact method of action.

Studies with 5% minoxidil applied topically have found minimal side effects.

According to pioneering research by Pharmacia and Upjohn, 26 percent of men between 18 and 49 reported from moderate to dense regrowth of normally pigmented hair fibers after four months of treating with minoxidil.



Inhibition of the 5-alpha-reductase enzyme, types I and II, by verbascoside (dark bars) and serenoa repens (light bars).

Another 33 percent reported they experienced at least some hair regrowth.

Almost 20 percent of women between 18 and 45 reported moderate hair regrowth, while another 40 percent showed some.

The researchers did not count vellus hairs, the short, thin, unpigmented strands that appear like peach fuzz.

In addition to minoxidil, Spectral.DNC-L incorporates an army of auxiliary agents to boost its effectiveness, plus other compounds selected to work at the hairline.

These multiple methods of action mean that Spectral.DNC-L works better than minoxidil alone, and works for patients who do not respond to minoxidil alone.

Arginine helps form nitric oxide to feed and regulate follicle activity.

AS A TRUE PIONEER, Spectral.DNC-L® incorporates arginine to strengthen and feed hair follicles because the amino acid is a metabolic precursor of nitric oxide, which is an important vasodilator and mediator of skin cell function.

Nitric oxide, a poisonous gas synthesized by the oxidation of nitrogen or ammonia, exists throughout the atmosphere and within the bodies of mammals. Key to optimizing nitric oxide within hair follicles, arginine is a safe and creative alternative to using the gas itself.

Chemical pathways for nitric oxide exist within

each hair cell. Increasing the volume of intracellular nitric oxide has been shown to increase the size and section of the follicle.

In one method of action, nitric oxide increases the blood flow to hungry hair follicles by relaxing vessels and causing dilation, helping hair to receive proper nutrition.

Through another method of action, it functions as an important mediator in various physiological and pathophysiological processes of the cutaneous system, such as regulating blood flow, melanogenesis, wound healing, and hyperproliferative skin diseases.

Exciting new research focuses on the role of nitric oxide in the human hair follicle and hair cycling. In 2003, a Berlin study demonstrated for the first time that human papilla cells produced

FREQUENTLY ASKED QUESTIONS

Q: How do **male hormones** affect hair loss?
A: Dihydrotestosterone seems to choke follicles by interfering with the enzyme adenylate cyclase, thus increasing sebum and decreasing hair growth.

Q: How do **nanosomes** boost effectiveness?
A: Compared to regular lotions, proprietary nanosome encapsulation delivers ingredients deeper into the scalp, where they can perform over time for maximum effect.

Q: How do **procyanidins** help to grow hair?
A: Procyanidin oligomers (polymers) support articulation of epithelial with mesenchymal cells, which induces anagen-phase growth.

the substance. Researchers believe it to be a signaling molecule, and basal- and androgen-mediated nitric oxide production to be involved in regulation of hair follicle activity.

The semiessential amino acid arginine, a precursor to nitric oxide, plays an important role in cell division, wound healing, and the immune function. Creatine formation also requires the chemical to stimulate protein synthesis. It helps to prevent wasting in people with critical illnesses. Symptoms of arginine deficiency include hair loss, skin rash, and poor wound healing.

In Spectral.DNC-L, the creative addition of arginine means improved blood flow and nutrition to follicles, as well as better overall regulation of the follicles' activity, so they can grow hair fibers of greater diameter.

FREQUENTLY ASKED QUESTIONS (CONTINUED)

Q: How does **Aminexil** help to grow hair?
A: Aminexil prevents the premature aging that results from rigidification of collagen.

Q: How does **biotin** help to grow hair?
A: Absorbed by the scalp, biotin penetrates the hair shaft directly, expanding it and thickening the cuticle.

Q: How does DS Laboratories get **herbal extracts**?
A: DS Laboratories uses gentle mechanical compression, not harsh chemical extraction, to preserve the molecular integrity and bioactivity of extracts.

Q: How does **research-grade minoxidil** contribute to Spectral.DNC-L?

Phytosterol 5-alpha-reductase inhibitors control hair-loss hormone.

SPECTRAL.DNC-L® deploys three potent compounds that inhibit the activities of 5-alpha-reductase, because that enzyme converts testosterone into dihydrotestosterone (DHT), which damages hair follicles. These key herbal extracts are olive (standardized with verbascoside), saw palmetto (serenoa repens), and flax seed (standardized with secoisolariciresinol diglucoside). Proprietary nanosomes deliver the inhibitors deep within the scalp.

In patients sensitive to DHT, closely associated with male pattern baldness, the hormone seems

FREQUENTLY ASKED QUESTIONS (CONTINUED)

A: Minoxidil was the first drug approved by the US Food and Drug Administration to treat male pattern baldness, and remains the most widely used topical compound today, although its exact method of action remains under study.

Q: How does **retinol** help to grow hair?
A: For hair growth, retinol controls the proper functioning of sebaceous glands.

Q: How does Spectral.DNC-L help to mitigate the damaging effects of **androgens**?
A: Spectral.DNC-L inhibits the action of 5-alpha-reductase, thus limiting dihydrotestosterone, sebum, and hair loss.

to choke follicles by interfering with the enzyme adenylate cyclase. This disruption increases sebum production and decreases hair growth. When the hormonal concentrations become high, thick terminal hairs whither and become thin vellus hairs (peach fuzz).

Spectral.DNC-L strongly inhibits 5-alpha-reductase, limiting DHT, sebum, and hair loss.

The best inhibitor, olive extract standardized with its active ingredient, verbascoside, blocks 5-alpha-reductase for healthy sebum regulation. Ripe olives contain biophenols, a class of molecules known for their antioxidant, anti-inflammatory, and antiviral properties. A major component of the biophenols, verbascoside inhibits 5-alpha-reductase type 2 at a rate 3.5 times greater than the second compound, serenoa repens.

FREQUENTLY ASKED QUESTIONS (CONTINUED)

Q: How effective is **Aminexil** for growing hair?
A: After minoxidil, Aminexil is the medication most widely studied and proven to preserve hair, protect roots, and strengthen fibers.

Q: How long should I treat to **get results**?
A: Treat consistently for three months, without missing applications, before evaluating results.

Q: How long should I treat in order to **keep my new hair**?
A: For androgenic alopecia, treatment must continue to retain your new hair. Otherwise, follicles would regress in two or three months.

The berries of saw palmetto (serenoa repens) contain lipids with a peculiar mix of fatty acids, alcohols, and phytosterols (plant-based steroids). Saw palmetto works to support hair growth by inhibiting 5-alpha-reductase and DHT. Its method of action is somewhat similar to the anti-alopecia drug finasteride (Propecia), and now generates a new wave of research on saw palmetto for preventing hair loss.

The third 5-alpha-reductase inhibitor, flax seed extract standardized with secoisolariciresinol diglucoside, is a nonsteroidal compound with activity similar to estrogen.

The inclusion of these three natural 5-alpha-reductase inhibitors means that Spectral.DNC-L works energetically to control damaging DHT, so hair follicles remain healthy.

FREQUENTLY ASKED QUESTIONS (CONTINUED)

Q: How was **minoxidil discovered**?
A: Minoxidil was given orally as a vasodilator to treat high blood pressure when researchers noticed new hair growth on test subjects.

Q: How would Spectral.DNC-L work even if **minoxidil alone** did not?
A: Multiple compounds and methods of action mean Spectral.DNC-L works for patients who do not respond to minoxidil alone.

Q: What are **procyanidins**?
A: Procyanidins are flavanols from apples. Current research indicates that they may restore the hairline as effectively as minoxidil restores the vertex.

Procyanidins promote hair growth through alternative methods of action.

SPECTRAL.DNC-L® contains procyanidins, flavanols derived naturally from apples, because the latest research indicates they restore the hairline nearly as effectively as minoxidil restores the vertex of the scalp.

Epithelial cells of a hair follicle include the outer root sheath, inner root sheath, and bulb matrix. The dermal papilla, however, is a mesenchymal cell, more like bone and connective tissue. Hair growth relies on successful interaction between these two cell types.

Procyanidin oligomers (simple polymers) pro-

FREQUENTLY ASKED QUESTIONS (CONTINUED)

Q: What do **herbal extracts** contribute to new hair growth?
A: Herbal extracts of ivy and hops promote blood circulation for follicle nutrition, scavenge free radicals to reduce cell damage, block dihydrotestosterone to slow hair loss, and oppose the proliferation of harmful bacteria.

Q: What does Spectral.DNC-L use as a **carrier agent**?
A: Ingredients are delivered deep into the scalp through proprietary nanosome encapsulation. The formula contains more extracts to soothe and condition skin and less filler.

mote both epithelial and mesenchymal cell health, which induces anagen-phase growth. Such stimulation may be all that is required to overcome some androgenetic alopecia.

Research compared hair regrowth after 19 days using procyanidin B-2, minoxidil, or placebo. Hair on the backs of mice was shaved and a topical solution applied daily. The effectiveness of procyanidin and minoxidil were equivalent for inducing anagen-phase hair growth.

After promising results with mice, Japanese labs conducted a human study. A double-blind clinical trial investigated the effects of a 1% procyanidin B2 solution over four months. Nineteen men received procyanidin; 10 received a placebo.

In the procyanidin group, 79 percent showed greater hair diameter, compared to 30 percent in

FREQUENTLY ASKED QUESTIONS (CONTINUED)

Q: What is the mission of **DS Laboratories**?
A: DS Laboratories exists to develop the highest-performing dermatological formulas.

Q: What is the role of **adenosine** for hair growth?
A: Adenosine facilitates hair growth because it works in biochemical processes like energy transfer and signal transduction.

Q: What is the role of **arginine** for hair growth?
A: Arginine is a safe way to optimize nitric oxide formation within hair follicles.

Q: What is the role of **nitric oxide** for hair growth?
A: Nitric oxide increases blood flow and nutrition for

the placebo group. The treated group also showed a higher ratio of hairs over 40 micrometers in diameter and higher total number of hairs.

This promising research means Spectral.DNC-L, which includes both procyanidin B-2 and C-1 in concentrations greater than the 1% tested, utilizes multiple methods of action to restore the hairline.

Adenosine facilitates energy transfer, signal transduction, and anti-inflammation.

THE ADENOSINE nucleoside plays an important role in hair growth because it works in biochemical processes like energy transfer (adenosine triphosphate, adenosine diphosphate) and signal

FREQUENTLY ASKED QUESTIONS (CONTINUED)

hair follicles by relaxing vessels, causing dilation, and other methods of action.

Q: What is the role of **ripe olive extract** for hair growth?
A: Verbascoside, the active ingredient from ripe olive extract, blocks 5-alpha-reductase to promote healthy sebum regulation.

Q: What is the role of **saw palmetto** for hair growth?
A: Saw palmetto inhibits 5-alpha-reductase and thus dihydrotestosterone by a method of action somewhat similar to the drug finasteride.

transduction (cyclic adenosine monophosphate).

The potent anti-inflammatory is under study for hair growth and wound healing. For androgenetic alopecia, adenosine proved promising in recent investigations conducted in Tokyo and Yokohama. On the theory that adenosine up-regulates the expression of fibroblast growth factor 7 and vascular endothelial growth factor on cultured dermal papilla cells, investigators assessed 101 volunteers in a randomized double-blind trial, comparing an adenosine 0.75% topical lotion against one with niacin amide 0.1%.

They applied the lotions twice daily for six months, then evaluated scalp coverage and the ratio of vellus to thick hairs. For increasing the ratio of thick hairs, as well as for global improvement, adenosine proved superior to niacin amide,

FREQUENTLY ASKED QUESTIONS (CONTINUED)

Q: What is the role of **vitamins and minerals** for hair growth?
A: The vitamin and mineral complex developed for Spectral.DNC-L supports the energy transfer and metabolic functions of hair follicles.

Q: When do I **treat with Spectral.DNC-L**?
A: Each morning and night, dispense four pumps of Spectral.DNC-L onto the fingertips to cover the affected area.

Q: When is **retinol prescribed** for hair?
A: Retinol, a fat-soluble form of vitamin A, improves the absorption and effectiveness of minoxidil. They are often prescribed together.

producing a higher ratio of thick hairs and lower ratio of vellus hairs. Side effects were not found.

This emerging science means that adenosine in Spectral.DNC-L stimulates growth without side effects, so users can restore both hair and lifestyle.

Retinol improves absorption and effects of active hair-growth compounds.

SPECTRAL.DNC-L® incorporates retinol, a fat-soluble form of vitamin A, because it improves the absorption and effectiveness of minoxidil. Often prescribed together, dermatological researchers have found that coupling retinol with minoxidil yields better results than either alone.

FREQUENTLY ASKED QUESTIONS (CONTINUED)

Q: When should I **begin treatment**?
A: Start as soon as symptoms appear because all treatments work better in the early stages of baldness, before follicles atrophy.

Q: Who has **tested minoxidil**?
A: Thousands of volunteers in hundreds of clinical trials have proven that minoxidil regrows hair on the vertex of the scalp.

Q: Who should **not use** Spectral.DNC-L?
A: If you have low blood pressure or take medication that depresses blood pressure, consult a physician before using Spectral.DNC-L.

Vitamin A derivatives get absorbed by the skin directly, increasing its turnover rate and building its collagen for more youthful appearance. The antioxidant vitamin maintains epithelial cell types (membranes) as a physical barrier against infection, builds immune system cells, including B-cells, T-cells, and natural killer cells, and affects production of human growth hormone.

For hair, retinol's greatest effect is to control the functioning of sebaceous glands, which produce sebum, a complex mixture of lipids that reduces water loss and protects the skin from infection.

But sebum also contains dihydrotestosterone (DHT), the hormone responsible for baldness. Moderating sebaceous glands can lower DHT.

People ingest retinol in its precursor form. Animal sources contain retinyl esters. Plants contain

FREQUENTLY ASKED QUESTIONS (CONTINUED)

Q: Who **should use** Spectral.DNC-L?
A: The most comprehensive formula available today, Spectral.DNC-L is indicated primarily for men who suffer from advanced androgenic alopecia — also called male pattern baldness — of 4 or greater on the Norwood scale.

Q: Why does Spectral.DNC-L **shift color**?
A: Natural apple polyphenol (procyanidin B-2) may change the color of the product from batch to batch, or cause the cream to darken with exposure to air, both of which are normal.

Q: Why does Spectral.DNC-L work **more effectively** than minoxidil alone?

provitamin A carotenoids such as beta-carotene.

Topical Spectral.DNC-L applies retinol directly to the site of follicular challenge, so it enhances the performance of minoxidil and other growth agents to help prevent and reverse hair loss.

Vitamin/mineral complex boosts metabolic function of hair follicles.

SPECTRAL.DNC-L® contains a rich vitamin and mineral complex, especially biotin and mineral salts, because these chemicals support energy transfer and the metabolic function of follicles.

A leading theory of hair loss is that oily sebum, laden with the hormone DHT, suffocates roots

FREQUENTLY ASKED QUESTIONS (CONTINUED)

A: Spectral.DNC-L brings together a broad spectrum of hair-growth compounds proven effective in clinical trials, working against hair loss through multiple methods of action.

Q: Why is **5-alpha-reductase** a problem?
A: The enzyme 5-alpha-reductase converts testosterone into dihydrotestosterone, which damages follicles and leads to hair loss. Spectral.DNC-L incorporates three effective natural inhibitors of 5-alpha-reductase.

and causes hairs to fall out. Physical and emotional stress exacerbates this loss by disturbing hormonal balance and depleting essential vitamins and minerals. The body responds by producing more hormones, stimulating more sebum, and causing more hairs to fall out.

Biotin in Spectral.DNC-L helps to break this damaging cycle by improving the metabolism of sebum. Dermatologists often prescribe this form of B vitamin — essential for hair, skin, and nail growth — for patients in treatment for hair loss.

Biotin plays a key role in nature's hair manufacturing process. Absorbed by the scalp, biotin penetrates the hair shaft directly, making it expand and thicken the cuticle.

The vitamin assists in production of fatty acids and metabolism of fats and amino acids. It plays a

critical role in the Krebs cycle, a sequence of reactions in which oxidation of acetic acid provides energy for storage in phosphate bonds, such as in adenosine triphosphate.

The key role of vitamins and minerals in skin health means they are also key ingredients in Spectral.DNC-L, so hair remains thick and strong.

Herbal extracts boost circulation and nutrition for targeted tissues.

THE PURE EXTRACTS of ivy and hops contained in Spectral.DNC-L stimulate the effectiveness of the minoxidil, Aminexil, and other active hair-growth agents in the formula, because these com-

pounds speed blood circulation and deliver cellular nutrition to affected tissues.

Applied topically, they penetrate the scalp near the roots, promoting blood circulation, scavenging free radicals, blocking DHT, and opposing harmful bacteria.

Hops have especially well-established medicinal uses and well-studied chemical properties. Two of their active ingredients are humulene, found in the essential oils, and lupuline, an alkaloid extracted as a colorless volatile liquid. Dried female buds of hops contain methylbutenol.

These natural herbal assistants mean that the active hair-growth agents in Spectral.DNC-L can work more effectively, so weaker hairs can get stronger faster.

DIRECTIONS FOR USE

Massage Spectral DNC-L into the scalp each morning and night. Dispense four (4) pumps of Spectral DNC-L onto the fingertips to cover the affected area completely. Massage it evenly into the scalp, then immediately wash hands with soap and water. Allow Spectral.DNC-L to dry completely before styling.

Treat consistently for three months, without missing, before evaluating results. Continue treatment to retain your new hair. For a more intensive start, especially the first three months after years of balding, add a mid-day treatment of Spectral.DNC®. This spray, with a complementary formula, acts through additional pathways for a broader attack on balding.

Aminexil® strengthens roots to prevent hair from falling out.

THE PATENTED molecule Aminexil makes Spectral.DNC-L so clearly superior to other hair-growth products because, after minoxidil, Aminexil is the medication most clearly proven to protect and grow hair.

In clinical trials, it preserves hair, protects roots, and strengthens fibers. The ingredient emerged from 10 years of proprietary research conducted by L'Oreal Laboratories.

Hair loss often results when roots become rigid, then compress the blood vessels that nourish them, and cause strands to fall out. With andro-

genic alopecia (male pattern baldness), the problem starts at the hairline and vertex, then spreads. Hairs get progressively finer, with a shorter life span, until they disappear altogether.

Aminexil, an innovation in the fight against baldness, prevents this premature aging, which

CONTRAINDICATIONS

This formulation is contraindicated in individuals with a history of sensitivity reactions to any of its components.

It should be discontinued if hypersensitivity to any of its ingredients is noted.

results technically from the rigidification of collagen. Once captured by the hairs' roots, the Aminexil preserves the suppleness and elasticity of the tissues that surround them. This method of action helps to build new hair fibers thicker, denser, and stronger.

In a trial with 120 men, hair loss began to slow after as little as three weeks. Hair also became softer to the touch. In a trial against placebo with 130 men, an additional eight percent of hair was maintained in growth phase after six weeks of Aminexil use.

These clinical study results indicate that Spectral.DNC-L, with Aminexil, preserves the vital functions of roots, so they can build more and stronger hair fibers.

GENERAL POINTS

- As with all topical hair loss medications, it is important to start treatment early. Since the treatment works by revitalizing hair follicles, ones that have been dormant for many years will not respond as well as ones that became compromised recently.

- Spectral.DNC-L will not prevent or improve hair loss due to medications, hypothyroidism, chemotherapy, or diseases that cause scarring of the scalp.

- For best results, apply Spectral.DNC-L directly to the scalp twice a day, every day, without skipping.

- In some patients, the product may cause scalp irritation. If so, stop using it and see a doctor.

INGREDIENTS AND COLOR

Spectral.DNC-L has Minoxidil, arginine, 5-alpha inhibitors, procyanidins, more.

ACTIVE ingredient: Minoxidil 5%. Other ingredients: Xanthan gum; PEG-100; isopropyl palmitate; octadecyl alcohol; hexadecanol; glycerin monostearate; white camphor oil; oleanolic acid, silicon oil; propylene glycol; proprietary nanosome dispersion of verbascoside, flax seed extract standardized with secoisolariciresinol diglucoside, and serenoa repens (saw palmetto) extract; procyanidin B-2 and C-1; adenosine, retinol; vitamin and mineral complex; ivy (hedera helix);

GENERAL POINTS (CONTINUED)

- Results may occur at two months with twice-a-day use. Some may need to use for at least three months before they see results. Hair grows at 1–2 cm per month, so it takes time for results to show.

- When you begin Spectral.DNC-L, hair loss may increase for up to two weeks. This temporary increase indicates that you are shedding old hairs to begin growing new ones. If loss continues after two weeks, discontinue use and see your doctor.

- If hair loss is due to hereditary male pattern baldness, continue use of Spectral.DNC-L or loss will begin again. If due to other factors, it may be possible to discontinue treatment and maintain the new hair.

hops (humulus lupulus); diaminopyrimidine oxide (Aminexil), piractone olamine; emu oil; menthol; arginine; copper tripeptide, DMDMH; methyldibromo glutaronitrile; phenoxyethanol.

Carrier: Nanosome microspheres.

Color: Apple polyphenol (procyanidin B-2) may change color from batch to batch, or cause cream to darken with exposure to air, which is normal.

GENERAL POINTS (CONTINUED)

- Allow Spectral.DNC-L to dry completely before bed, to prevent smearing on the pillow, transferring the medication to the face, and causing unwanted facial hair growth.

- Apply 1 ml (four pumps) twice per day directly to areas of hair loss and thinning. Using less will reduce the effectiveness of the treatment.

- If you miss a dose of Spectral.DNC-L, do not apply multiple doses the next day. Just return to your normal treatment schedule.

- Spectral.DNC-L should not be used by people under 18 years of age.

CONTRAINDICATIONS

Pregnant women, nursing women, and coronary patients consult doctor first.

SPECTRAL.DNC-L® has vasodilators that increase absorption, so some users may experience reduced blood pressure. If you have low blood pressure or take medicine that depresses pressure, consult a physician before using. Patients with significant coronary disease or history of heart failure, and women who may be pregnant or nursing, should not use the product. If you experience rapid heartbeat, dizziness, or shortness of breath, discontinue use and seek medical attention immediately.

PUBLISHED SCIENCE

Peer-reviewed medical journal studies examine ingredients in Spectral.DNC-L

OVER THE NEXT several pages, read what the leading medical journals in dermatology have published about the cutting-edge ingredients incorporated into Spectral.DNC-L, plus the general issue of androgenic alopecia (male and female pattern baldness).

AG Messenger, J Rundegren (2004). Minoxidil: Mechanisms of action on hair growth. *British Journal of Dermatology* 150 (2), 186-194.
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REVIEW ARTICLE

Minoxidil: mechanisms of action on hair growth

A.G.MESSENGER AND J. RUNDEGREN*
Department of Dermatology, Royal Hallamshire Hospital, Sheffield S10 2JF, U.K. *Pharmacia Consumer Healthcare, Box 941, SE-251 09 Helsinborg, Sweden
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Summary

We have known for over 30 years that minoxidil stimulates hair growth, yet our understanding of its mechanism of action on the hair follicle is very limited. In animal studies, topical minoxidil shortens telogen, causing premature entry of resting hair follicles into anagen, and it probably has a similar action in humans. Minoxidil may also cause prolon-

gation of anagen and increases hair follicle size. Orally administered minoxidil lowers blood pressure by relaxing vascular smooth muscle through the action of its sulphated metabolite, minoxidil sulphate, as an opener of sarcolemmal K_{ATP} channels. There is some evidence that the stimulatory effect of minoxidil on hair growth is also due to the opening of potassium channels by minoxidil sulphate, but this idea has been difficult to prove and to date there has been no clear demonstration that K_{ATP} channels are expressed in the hair follicle. A number of in vitro effects of minoxidil have been described in monocultures of various skin and hair follicle cell types including stimulation of cell proliferation, inhibition of collagen synthesis, and stimulation of vascular endothelial growth factor and prostaglandin synthesis. Some or all of these effects may be relevant to hair growth, but the application of results obtained in cell culture studies to the complex biology of the hair follicle is uncertain. In this article we review the current state of knowledge on the mode of action of minoxidil on hair growth and indicate lines of future research.

Key words: androgenetic alopecia, hair, minoxidil

Minoxidil was introduced in the early 1970s as a treatment for hypertension. Hypertrichosis was a common side-effect in those taking minoxidil tablets[1,2] and included the regrowth of hair in male balding.[3] This led to the development of a topical formulation of minoxidil for the treatment of androgenetic alopecia in men and subsequently in women. The 2% product was first marketed for hair regrowth in men in 1986 in the United States and the 5% product became available in 1993.

Despite much research over 20 years we still have only a limited understanding of how minoxidil stimulates hair growth. Nevertheless, understanding minoxidil's mechanism of action is important, both from the point of view of developing more effective treatments for hair loss disorders and for the insights it may give into the biology of hair growth. In this article we review what is known about the pharmacology of minoxidil, with particular reference to its action on hair growth, and suggest directions for future research.

Response of the hair follicle to minoxidil

There are a number of ways in which a drug may stimulate hair growth; it may increase the linear growth rate of hair,

increase the diameter of the hair fibre, alter the hair cycle, either shortening telogen or prolonging anagen, or act through a combination of these effects. Present evidence suggests that minoxidil acts mainly on the hair cycle; it may also increase hair diameter.

Animal studies

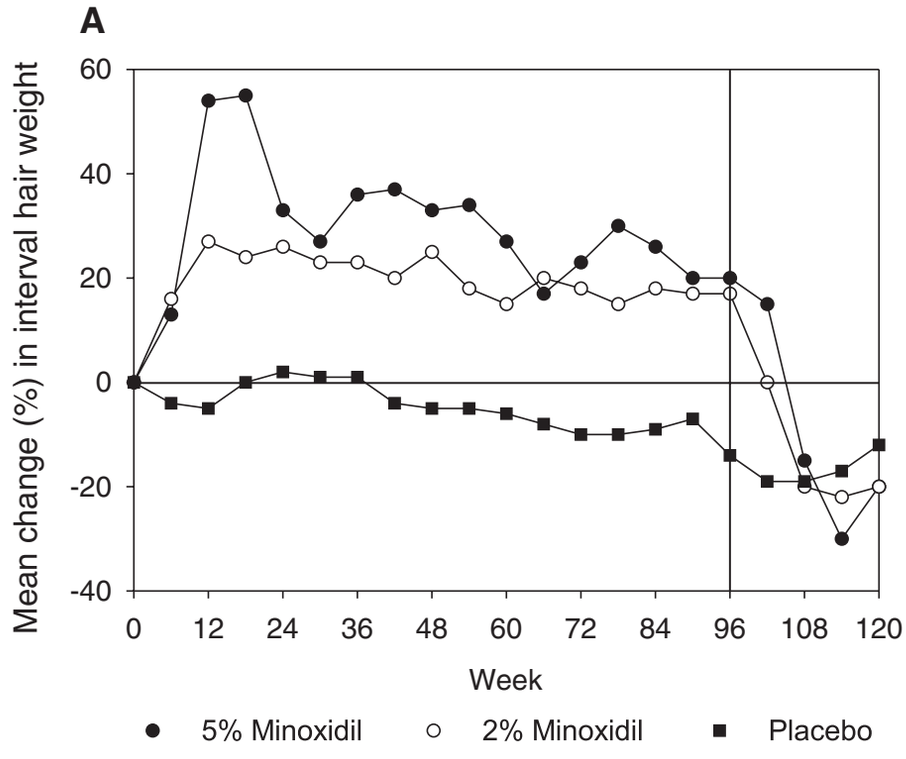
Mori and Uno[4] studied the effect of topical application of minoxidil on spontaneous hair cycles in the rat from birth to 80 days of age. Minoxidil had no effect on the duration of anagen, but telogen was shortened. The telogen phase of the third cycle lasted approximately 20 days in untreated animals, whereas follicles re-entered anagen after only 1–2 days in telogen in minoxidil-treated animals. The same shortening of telogen by minoxidil treatment was also seen in the fourth cycle. The effect of minoxidil on hair growth has been studied extensively in the stump-tailed macaque, a primate that develops postadolescent scalp hair loss closely resembling human androgenetic alopecia. Topical minoxidil prevents the development of scalp hair loss in peri-adolescent macaques and promotes regrowth of hair in balding animals. Histological studies showed that treatment with minoxidil causes an increase in the proportion of fol-

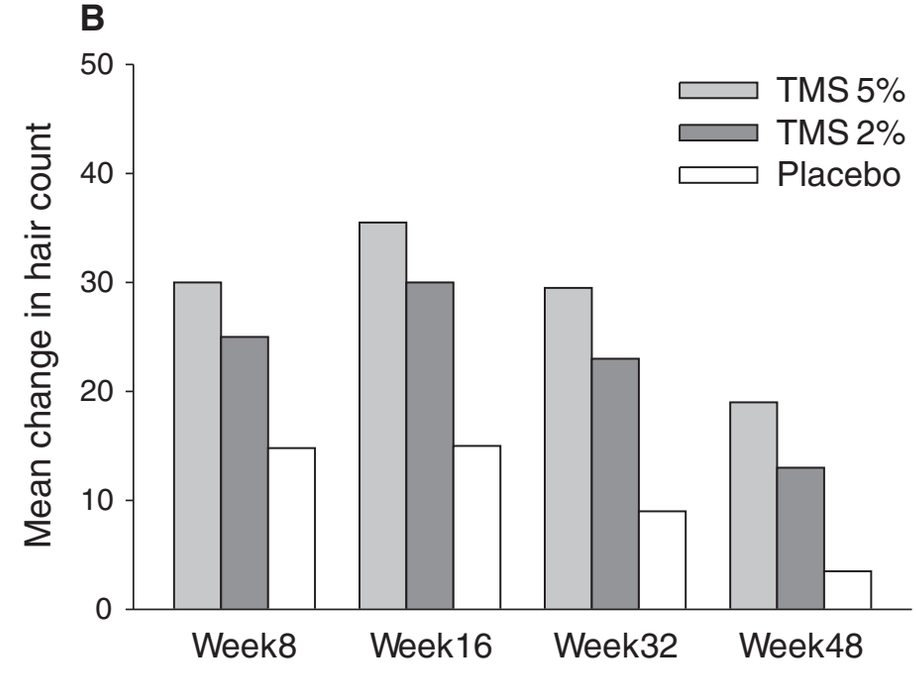
licles in anagen, a reduction in telogen follicles, and an increase in hair follicle size.[5]

Humans

Little is known of the effect of minoxidil on normal human hair growth and studies have been limited mainly to the response of androgenetic alopecia to topical minoxidil. In male pattern balding (male androgenetic alopecia) there is a gradual reduction in the duration of anagen and a prolongation

Figure 1. Results of two clinical trials of minoxidil topical solution in the treatment of male androgenetic alopecia using different methods for measuring the response. Both methods show a rapid increase in hair growth which has reached a plateau by 12–16 weeks. (A) Comparison of mean percentage change in interval hair weight per square centimetre for three treatment groups: 5% minoxidil, 2% minoxidil and placebo. Vertical line at 96 weeks indicates cessation of treatment. Adapted from Price et al.[65] (B) Mean change from baseline in nonvellus hair counts (per square centimetre) in men treated with 5% minoxidil solution (TMS), 2% minoxidil and placebo. From Olsen et al.[66]


A
Mean change (%) in interval hair weight
60
40
20
0
-20
-40
0
12
24
36
48
60
72
84
96
108
120
Week
5% Minoxidil
2% Minoxidil
Placebo


B
Mean change in hair count
50
40
30
20
10
0
TMS 5%
TMS 2%
Placebo
Week8
Week16
Week32
Week48

of the latent period of the hair cycle (the time between shedding of the telogen hair and the onset of the next anagen).[6] Hair follicles also become miniaturized.[7] There is some controversy over whether female androgenetic alopecia is the same entity as male balding. Nevertheless, the follicular changes are very similar,[8,9] if not identical, although prolongation of the latent period has not yet been demonstrated in women. Clinical trials of topical minoxidil in male and female hair loss all show a remarkably rapid increase in hair growth, measured by hair counts or hair weight. The increase is evident within 6–8 weeks of starting treatment and has generally peaked by 12–16 weeks (Fig. 1). It seems improbable that a response of this rapidity can be accounted for by reversal of follicular miniaturization, and a more likely explanation is that minoxidil triggers follicles in the latent part of telogen into anagen. The hypertrichosis that develops in humans taking minoxidil orally, and occasionally following topical use, may affect the forehead as well as other sites such as the limbs. The increased length of hair at these sites suggests that minoxidil also prolongs the duration of anagen in humans.

The results of histological studies in humans are less conclusive than in the macaque. Abell[10] found a trend towards

an increase in anagen/telogen ratios after 12 months of minoxidil treatment in balding men, but the main change was an increase in mean hair diameter. This was most apparent at 4 months and mean diameter had declined at 12 months. He suggested this might be due to later recruitment of small diameter hairs into anagen. Headington and Novak[11] reported that minoxidil treatment caused hypertrophy of follicles but, although there was an increase in mean hair diameter in minoxidil-treated balding men after 12 weeks, a similar increase occurred in control subjects. Care should be taken in interpreting change in mean hair diameters. This does not necessarily imply that individual hair follicles become larger, as an increase in mean diameter may also occur through preferential recruitment of large diameter hairs in a latent phase of the hair cycle.

Minoxidil sulphation

The antihypertensive activity of minoxidil is due to rapid relaxation of vascular smooth muscle by its sulphated metabolite, minoxidil sulphate.[12,13] The conversion of minoxidil to minoxidil sulphate is catalysed by sulphotransferase enzymes. Minoxidil sulphotransferase activity was initially

demonstrated in rat liver[12] and has since been found in human liver,[14] platelets[15] and epidermal keratinocytes,[16] as well as in mouse vibrissae follicles,[17] rat pelage and vibrissae follicles and rat epidermal keratinocytes.[18,19] In scalp skin of stumptail macaques, sulphotransferase activity is largely localized in the hair follicle.[20] In rat pelage and vibrissae follicles, immunoreactivity for minoxidil sulphotransferase was seen in the outer root sheath.[18] Five human cytosolic sulphotransferase genes have been discovered to date. They encode three classes of enzymes responsible for sulphating phenols and catecholamines, oestrogens and hydroxysteroids.[21] In human liver extracts, sulphation of minoxidil is catalysed by at least four sulphotransferases. Biochemical evidence for minoxidil sulphation by two phenol sulphotransferases has been found in human scalp skin[22] and Dooley[21] reported finding mRNA expression for four sulphotransferases in human epidermal keratinocytes. There are interindividual variations in scalp sulphotransferase activity and this correlates with the level in platelets.[22] In a clinical setting, scalp sulphotransferase activity was higher in men who responded to minoxidil compared with those who did not respond.[23]

Minoxidil sulphate is a potassium channel opener

Minoxidil sulphate is one of several chemically unrelated drugs which cause opening of plasma membrane adenosine triphosphate (ATP)-sensitive potassium channels (K_{ATP} channels), and its relaxant effect on vascular smooth muscle is mediated through this mechanism.[24,25] K_{ATP} channels are heteromultimers composed of a small subunit that belongs to the inwardly rectifying potassium channel superfamily ($K_{IR}6.1$ or $K_{IR}6.2$), and a large sulphonylurea receptor (SUR1, SUR2A or SUR2B) that binds sulphonylureas and ATP and belongs to the ATP-binding cassette (ABC) superfamily.[26] SUR1/$K_{IR}6.2$ K_{ATP} channels are found in pancreatic and neuronal tissue, whereas SUR2A/$K_{IR}6.2$ and SUR2B/$K_{IR}6.1$ (or $K_{IR}6.2$) form the cardiac and vascular smooth muscle K_{ATP} channels, respectively. Potassium channel openers act through binding to the sulphonylurea receptor moiety.[26]

K_{ATP} channels are widely distributed in a variety of tissue and cell types, including cells of the heart, pancreas, vascular smooth muscle and the central nervous system, where they couple intracellular metabolic changes to the electrical activity of the plasma membrane.[27] These potassium channels sense the metabolic state of the cell — channel opening is

inhibited by ATP when energy levels are high and is activated when energy stores are depleted.[28] The consequence of K_{ATP} status depends on the cell and tissue type. For example, in pancreatic β cells, K_{ATP} channels are involved in regulating insulin secretion. In vascular smooth muscle cells the vasodilating action of potassium channel openers is due to membrane hyperpolarization and a reduction in Ca^{2+} influx, which reduces the electrical excitability of the cell. It has also been suggested that potassium channel activity is required for early-stage cell proliferation by G_1 progression of the cell cycle.[29] Minoxidil was shown to increase DNA synthesis, whereas glibenclamide suppressed DNA synthesis in rat primary hepatocyte cultures.[30] Hepatocyte potassium currents were augmented by minoxidil and attenuated by glibenclamide.

Does minoxidil act on hair growth via potassium channels?

Several lines of evidence, from clinical observations, animal studies and in vitro experiments, suggest that the promotion of hair growth by minoxidil is related in some way to its action as a potassium channel opener (Table 1).

Table 1. Does minoxidil act on hair growth via potassium channels?

Evidence for

1. Chemically unrelated potassium channel openers stimulate hair growth:
   a. in humans (minoxidil, diazoxide, pinacidil)
   b. in macaques (minoxidil, cromakalin, P-1075)
2. Chemically unrelated potassium channel openers stimulate thymidine and/or cysteine uptake by mouse vibrissae follicle in vitro (minoxidil, pinacidil, cromakalin, nicorandil, P-1075, diazoxide)
3. Stimulation of 3T3 fibroblast proliferation by minoxidil in vitro inhibited by potassium channel antagonists (tolbutamide, tetraethylammonium).

Evidence against

1. Stimulation of thymidine/cysteine uptake by minoxidil in cultured mouse vibrissa follicles not blocked by potassium channel antagonists.
2. 86Rb efflux in vibrissae follicle cultures not increased by minoxidil.
3. KATP channels not demonstrated in cultured hair follicle cells by patch-clamp methods.

In vivo studies

In addition to minoxidil, the potassium channel openers diazoxide[31,32] and pinacidil[33] cause hypertrichosis in humans. Buhl et al.[34] tested the effect of topical application of minoxidil and three other potassium channel openers on scalp hair growth in balding macaques. Minoxidil, cromakalin and P-1075 (a pinacidil analogue) all stimulated hair growth over a 20-week treatment period. A fourth potassium channel opener, RP-49,356, was not effective.

Organ culture studies

Buhl et al.[35] carried out a series of experiments on minoxidil action using cultured mouse vibrissae follicles. In 3-day cultures, 1 mmol L^{-1} minoxidil preserved follicular morphology, whereas follicles cultured in the absence of minoxidil degenerated rapidly. Follicles cultured in 0.5–5 mmol L^{-1} minoxidil grew longer than controls and showed higher levels of uptake of radiolabelled cysteine, amino acids and thymidine. This effect appears to be mediated by minoxidil sulphate. The same results were obtained using approximately 100-fold lower concentrations of minoxidil sulphate and the response of cultured follicles to minoxidil, but not minoxidil sulphate, was blocked by diethylcarbamazine and

chlorate, agents which interfere with sulphation.[17] The potassium channels openers pinacidil, cromakalin, nicorandil and P-1075 also stimulated uptake of radiolabelled cysteine in cultured vibrissae follicles, although diazoxide did not.[36] Harmon et al.[37] also reported that minoxidil, pinacidil, cromakalin and diazoxide increased uptake of thymidine in a dosedependent fashion in 4-day cultures of mouse vibrissae follicles. These studies imply that minoxidil stimulates hair growth in this model by opening potassium channels, but attempts to verify this idea have been unsuccessful. The broad-spectrum ion channel blocker tetraethylammonium chloride and the K_{ATP} channel blockers, glyburide and tolbutamide, failed to inhibit minoxidil stimulation of cultured vibrissae follicles at doses that were not themselves toxic.[34] To test whether minoxidil opened ion channels, vibrissae follicles were labelled with $^{86}Rb^{+}$, an ion with specificity for potassium channels similar to K^{+}. In this model, the potassium channel opener pinacidil increased efflux of $^{86}Rb^{+}$ but minoxidil did not.[34]

Human hair follicle organ culture has been used extensively in hair biology but there is only a single published report describing increased uptake of thymidine by cultured

human hair follicles in response to minoxidil.[38] Minoxidil causes premature entry of follicles into anagen, and probably prolongs anagen and increases hair follicle size. Of these effects only the prolongation of anagen is possibly modelled by hair follicle organ culture and even here the alteration in follicle survival in vitro is measured in days rather than the weeks or months achieved in vivo. The rather mixed responses of cultured follicles to minoxidil may therefore be due to insensitivity or inapplicability of the model. However, minoxidil does prolong survival of cultured follicles that would otherwise undergo rapid degeneration in vitro, albeit at concentrations which are unlikely to be achieved in vivo. This effect appears to be mediated by the sulphated metabolite and there is circumstantial but, as yet, unconfirmed evidence that it involves opening of potassium channels.

Cell culture studies

Sanders et al. showed that the stimulatory effect of minoxidil on the growth of 3T3 fibroblasts is inhibited by pharmacological blockade of potassium channels.[39] As yet, however, there is no clear evidence that K_{ATP} channels are expressed in cells of hair follicle derivation. Nakaya et al. looked for potassium channels in cultured hair follicle outer root sheath

and dermal papilla cells using the patch-clamp technique.[40] They identified large and small conductance calcium-activated potassium channels in cell membranes. These channels were not blocked by ATP or glibenclamide (a specific K_{ATP} channel blocker) and neither minoxidil sulphate nor pinacidil increased efflux of ^{86}Rb, suggesting the absence of K_{ATP} channels. However, the same group has recently reported that human dermal papilla cells express mRNA for the sulphonylurea receptor SUR2B,[41] the same sulphonylurea receptor expressed in vascular smooth muscle cells.

The cellular response to minoxidil

Whatever the mechanism whereby minoxidil modulates hair growth, there must be a primary effect on cell function (Table 2). The hair follicle is a complex structure comprising epithelial, dermal, pigment and immune cells, and a perifollicular vasculature and neural network. Interactions between these cells are involved in regulating epithelial growth and differentiation and the hair cycle. Several of these cell types have been used in isolation to study minoxidil action, but attempts to localize minoxidil or a minoxidil metabolite binding to a specific cell population within the hair follicle

have been unsuccessful.[42] Uptake studies in mouse vibrissae follicles showed that minoxidil and minoxidil sulphate concentrated in melanocytes and pigmented epithelial cells in the suprapapillary region of the follicle. However, this was probably due to nonspecific binding to melanin as there was no evidence of minoxidil binding in nonpigmented follicles yet pigmented and nonpigmented follicles showed a similar growth response to minoxidil.[43]

Cell proliferation

Several studies have examined the effect of minoxidil on cell proliferation in vitro. A variety of cell types have been used including epidermal keratinocytes, hair follicle keratinocytes and skin fibroblasts from humans, mice and macaques. In some studies, established keratinocyte and fibroblast cell lines have been used. The results have been variable and, to some extent, contradictory.

Boyera et al.[44] studied the effect of minoxidil on human keratinocytes of epidermal and hair follicle origin using a range of different culture conditions and proliferative markers. They found that micromolar concentrations of minoxidil stimulated proliferation in both cell types and in all culture conditions, whereas millimolar concentrations inhibited cell

growth. In cells cultured from the stumptail macaque, minoxidil stimulated thymidine uptake by follicular keratinocytes but not by epidermal keratinocytes.[45] O'Keefe and Payne[46] also failed to show a stimulatory response to minoxidil in

Table 2. Effects of minoxidil on cell function

Cell growth	Variable effects on growth and survival of cells in culture. In different studies minoxidil has been reported to inhibit or stimulate growth of epithelial and fibroblast cell types. Delays senescence in keratinocyte cultures.
Collagen synthesis	Inhibits lysyl hydroxylase. Inhibits collagen production.
Prostaglandin synthesis	Stimulates PGE2 synthesis. Inhibits prostacyclin production.
VEGF	Stimulates VEGF synthesis by dermal papilla cells. Stimulation of VEGF synthesis mediated by adenosine.

VEGF, vascular endothelial growth factor.

cultured human epidermal keratinocytes, although Baden and Kubilus[47] reported that minoxidil prolonged the time after confluence that keratinocytes could be subcultured.

Studies using fibroblasts have yielded similarly variable results. Murad and Pinnell[48] reported that high concentrations of minoxidil inhibited growth of human skin fibroblasts. On the other hand, thymidine uptake was increased in macaque follicular fibroblasts cultured in micromolar concentrations of minoxidil, but not in nonfollicular fibroblasts.[45] Sanders et al.[39] proposed that the variable results of cell culture experiments may be explained by the potassium channel-blocking activity of aminoglycoside antibiotics, routinely incorporated into cell culture media. Minoxidil stimulated growth of NIH 3T3 fibroblasts cultured in the absence of aminoglycosides but not in their presence, and the proliferative response of 3T3 cells to minoxidil was prevented by the potassium channel blockers tolbutamide and tetraethylammonium. In cultured human keratinocytes, aminoglycoside antibiotics partly suppressed the proliferative response to minoxidil but did not abolish it.[44]

The variations in the cell types and experimental protocols used mean that it is difficult to compare the results from these

studies. On balance, they suggest that minoxidil can have a stimulatory effect on cell growth at clinically relevant concentrations, or delay cell senescence, and there is limited evidence that this is mediated by its action as a potassium channel opener.

Collagen synthesis

Two groups have studied the effect of minoxidil on collagen synthesis. Murad and Pinnell[48] showed that minoxidil suppressed activity of the enzyme lysyl hydroxylase in human skin fibroblast cultures at concentrations down to 25 μmol L^{-1}, leading to production of a collagen deficient in hydroxylysine.[49] This appeared to be specific for lysyl hydroxylase as the activity of prolyl hydroxylase, which shares the same substrates and cofactors as lysyl hydroxylase, was unaffected. Minoxidil (0.5 mmol L^{-1}) also suppressed collagen synthesis by rat vibrissae dermal papilla cells, both in monolayer cultures and in cells grown in collagen gels.[50] The concentrations of minoxidil used in these studies were quite high and the relevance of the results to hair growth is unknown.

Prostaglandins

The prostaglandin PGH_2 is formed from arachidonate by the action of a cyclooxygenase (COX), also known as pros-

taglandin endoperoxide synthase (PGHS). PGH_2 is the substrate for subsequent enzymatic modifications leading to the prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$), prostacyclin (PGI_2) and thromboxane A_2. There are two isoforms of PGHS, a widely distributed constitutive form PGHS-1, and an inducible form PGHS-2. The PGHS-1 isoform has been immunolocalized to the dermal papilla of human hair follicles during anagen and catagen.[51] Immunostaining for PGHS-2 was also seen in the dermal papilla but staining was weaker than that for PGHS-1 and was present only in anagen follicles. Minoxidil (AC50 = 80 μmol L^{-1}) stimulated the activity of purified ovine PGHS-1 in vitro and increased production of PGE_2 in cultured human dermal papilla cells and mouse fibroblasts. Lachgar et al.[50] also found that minoxidil (12 μmol L^{-1}) stimulated PGE_2 production by cultured dermal papilla cells, in this case derived from rat vibrissae, as well as production of leukotriene B_4. They also found that minoxidil inhibited prostacyclin synthesis by dermal papilla cells (measured as 6-keto-prostaglandin $F_{1\alpha}$), as had an earlier study using bovine endothelial cells.[52] Prostanoids have many biological functions in different tissues, acting through specific G protein-coupled receptors[53] and, in some cases, via nuclear receptors.[54] We do

not know whether prostanoids have a physiological role in regulating hair growth, although, latanoprost, a topical synthetic $PGF_{2\alpha}$ analogue used in the treatment of glaucoma, causes hypertrichosis of the eyelashes.[55] Topical treatment with latanoprost also stimulates hair regrowth on the scalp in balding stumptail macaques.[56]

Androgen responses

Nuck et al.[57] studied the antiandrogenic potential of minoxidil on androgen-dependent cutaneous structures of the flank organ of female golden Syrian hamsters. Neither 1% nor 5% minoxidil topical solution applied to one flank for 3 weeks prevented the androgen-dependent growth of the pigmented spot, sebaceous glands or hair follicle diameter induced by subcutaneous capsules filled with testosterone. However, significant inhibition was seen following topical application of 5% progesterone. The effect of minoxidil on human hair growth is not confined to androgen-dependent hair follicles and these findings are consistent with the conclusion that minoxidil does not act through androgen pathways. However, Sato et al.[58] reported that minoxidil stimulates 17β-hydroxysteroid dehydrogenase (17β-HSD) in cultured human dermal papilla cells and also has a small stimulatory effect on

5α-reductase activity. 17β-HSD catalyses the interconversion of testosterone and androstenedione and may therefore increase or reduce androgen responses. A high concentration of minoxidil (0.5 mmol L^{-1}) was used in this study and the relevance of the results to hair growth in vivo is unclear.

Vascular effects

The idea that minoxidil stimulates hair growth by increasing cutaneous blood flow has been the subject of two studies giving contradictory results. Wester et al.[59] studied the effect of topical minoxidil (1%, 3%, 5%) on blood flow in balding scalp using laser Doppler velocimetry (LDV) and photopulse plethymography. Both methods showed an increase in skin blood flow following application of minoxidil that was statistically significant with the 5% solution. On the other hand, Bunker and Dowd,[60] also using LDV, failed to find any change in skin blood flow following application of 3% minoxidil topical solution to the scalp in 10 balding men, whereas all but one showed an increase in blood flow after applying the vasodilator 0.1% hexyl nicotinate. The difference in results may have been due to the higher concentration of minoxidil used in the first study although, as Bunker and Dowd point out, 3% minoxidil topical solution is clinically effective.

Sakita et al.[61] studied the effect of minoxidil topical solution on the hair follicle vasculature in the rat using transmission electron microscopy. In minoxidil-treated animals there was no difference in the total area of follicular capillaries compared with controls but there was an increase in capillary fenestrations. The authors suggested that the increase in fenestrations may be due to vascular endothelial growth factor (VEGF) (see below), but the functional significance of this observation was not discussed.

Vascular endothelial growth factor

VEGF has a central role in promoting angiogenesis as well as influencing diverse cell functions including cell survival, proliferation and the generation of nitric oxide and prostacyclin.[62] The perifollicular capillary network is coupled to the hair cycle, increasing during anagen and then regressing during catagen and telogen. Yano et al.[63] found that capillary proliferation during anagen was temporally and spatially associated with expression of VEGF in the outer root sheath of murine hair follicles. Transgenic overexpression of VEGF in the outer root sheath increased perifollicular vascularization and led to accelerated hair growth following depilation and the growth of larger hairs. This effect was prevented by sys-

temic administration of a VEGF antibody. Lachgar et al.[64] found that the expression of VEGF mRNA and protein in cultured human dermal papilla cells was stimulated by minoxidil in a dose-dependent fashion. A fivefold increase in VEGF protein occurred in extracts of cells incubated with 12 μmol L^{-1} minoxidil, and there was a similar increase in mRNA expression. A possible mechanism for minoxidil stimulation of VEGF has been proposed by Li et al. from experiments on cultured dermal papilla cells.[41] They found that adenosine also increases VEGF release and the VEGF response to minoxidil was prevented by pharmacological blockade of A1 and A2 adenosine receptors. mRNAs for the A1, A2A and A2B adenosine receptors, as well as the sulphonylurea receptor SUR2B, were detected by the reverse transcriptase–polymerase chain reaction. The authors suggested that binding of minoxidil to SUR2B promotes secretion of ATP, which is rapidly converted to adenosine and activates adenosine signalling pathways.

Conclusions

The emergence of topical minoxidil for the treatment of androgenetic alopecia in the early 1980s led to the realization that hair loss is potentially treatable and ushered in a new

era in hair research. The series of experiments by Buhl and others on cultured vibrissae follicles and on the stumptail macaque support the view that the hair follicle response to minoxidil is mediated by its sulphated metabolite acting as a potassium channel opener. Nevertheless there are inconsistencies in the results that have yet to be resolved and this idea must be viewed as unproven. A variety of responses to minoxidil have been described in cultured cells. Some have potential relevance to hair growth, such as the effects on cell growth and senescence and the stimulation of VEGF and prostaglandin synthesis. Others, such as the effects on collagen synthesis, are more difficult to explain. Viewed in isolation, the results of cell culture studies must be interpreted with care. First, the relationship between the complexities of hair growth and the behaviour of a single cell type cultured in a Petri dish is uncertain. Second, the concentrations of minoxidil used have often exceeded those to which the hair follicle is likely to be exposed in vivo. Blood levels in subjects taking minoxidil orally are in the upper nanomolar/low micromolar range (20–2000 ng mL^{-1}) and are lower still in those using minoxidil topically (≈ 2 ng mL^{-1}). Third, the minoxidil target cell population in the hair follicle is unknown.

Nevertheless, the stimulation of VEGF and prostaglandin synthesis by minoxidil in dermal papilla cells provides an attractive and logical starting point for future studies and is backed up by evidence from other sources. We need to know more about the signalling mechanisms responsible for these effects — do they involve conventional potassium channel physiology or a novel mechanism as suggested by Li et al.?[41] Are K_{ATP} channels operating in the regulation of normal hair growth or the development of androgenetic alopecia and, if so, what is their subtype composition and cellular and subcellular distribution?

Why is minoxidil important? Although the benefits in androgenetic alopecia have been demonstrated in clinical trials, there is perhaps a tendency to dismiss the significance of minoxidil. Yet, it remains the only medical treatment of proven efficacy when used topically and is the only treatment approved for hair loss in women. Minoxidil affects hair cycling, causing premature termination of telogen and probably prolonging anagen. Understanding how minoxidil exerts these effects may lead not only to better treatments for hair loss but also will increase our understanding of the mechanisms responsible for controlling the hair cycle.

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Correspondence: A.G.Messenger.
E-mail: a.g.messenger@sheffield.ac.uk
Conflicts of interest: Dr Messenger is a dermatologist and has been a consultant for Pharmacia and other pharmaceutical companies with an interest in the field of hair growth. Dr Rundegren is an employee of Pharmacia.

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Minoxidil-Induced Hair Growth is Mediated by Adenosine in Cultured Dermal Papilla Cells: Possible Involvement of Sulfonylurea Receptor 2B as a Target of Minoxidil

Ming Li, Azusa Marubayashi,* Yutaka Nakaya,† Kiyoshi Fukui,* Seiji Arase.
Department of Dermatology, School of Medicine, *Division of Gene Regulatorics, The Institute for Enzyme Research, and †Department of Nutrition, School of Medicine, The University of Tokushima, Tokushima, Japan

The mechanism by which minoxidil, an adenosine-triphosphate-sensitive potassium channel opener, induces hypertrichosis remains to be elucidated. Minoxidil has been reported to stimulate the production of vascular endothelial growth factor, a possible promoter of hair growth, in cultured

dermal papilla cells. The mechanism of production of vascular endothelial growth factor remains unclear, however. We hypothesize that adenosine serves as a mediator of vascular endothelial growth factor production. Minoxidil-induced increases in levels of intracellular Ca^{2+} and vascular endothelial growth factor production in cultured dermal papilla cells were found to be inhibited by 8-sulfophenyl theophylline, a specific antagonist for adenosine receptors, suggesting that dermal papilla cells possess adenosine receptors and sulfonylurea receptors, the latter of which is a well-known target receptor for adenosine-triphosphate-sensitive potassium channel openers. The expression of sulfonylurea receptor 2B and of the adenosine A1, A2A, and A2B receptors was detected in dermal papilla cells by means of reverse transcription polymerase chain reaction analysis. In order to determine which of the adenosine receptor subtypes contribute to minoxidil-induced hair growth, the effects of subtype-specific antagonists for adenosine receptors were investigated. Significant inhibition in increase in intracellular calcium level by minoxidil or adenosine was observed as the result of pretreatment with 8-cyclopentyl-1,3-dipropylxanthine, an antagonist for adenosine A1 receptor, but not by

3,7-dimethyl-1-propargyl-xanthine, an antagonist for adenosine A2 receptor, whereas vascular endothelial growth factor production was blocked by both adenosine A1 and A2 receptor antagonists. These results indicate that the effect of minoxidil is mediated by adenosine, which triggers intracellular signal transduction via both adenosine A1 and A2 receptors, and that the expression of sulfonylurea receptor 2B in dermal papilla cells might play a role in the production of adenosine.

Key words: adenosine receptor / ATP-sensitive potassium channel/hypertrichosis/intracellular calcium/vascular endothelial growth factor. J Invest Dermatol 117:1594–1600, 2001

Minoxidil has been used for patients with severe hypertension who were refractory to other treatments (Dargie et al, 1977). The opening of the adenosine triphosphate (ATP) sensitive potassium channel (K_{ATP} channel), followed by the generation of membrane potential, results in a reduction of intracellular calcium ($[Ca^{2+}]i$) levels and in vascular smooth muscle relaxation, which is generally thought to contribute to this hypotensive effect (Andersson, 1992). In addition to this effect, minoxidil (Earhart et al, 1977; Burton

and Marshall, 1979), pinacidil, diazoxide, and other K_{ATP} channel openers (Burton et al, 1975; Goldberg, 1988) have been reported to cause hypertrichosis in humans, though the precise mechanisms by which these drugs induce hair growth remains unclear. It is known, however, that despite their diverse chemical structures they share a common feature; namely, their actions of K_{ATP} channel opening. Dermal papilla cells (DPC), which reside at the base of hair follicles, were highly implicated as the source of regulatory stimuli of hair growth and development. Therefore, K_{ATP} channel opening in DPC has been suggested as an important mechanism in terms of the ability of DPC to stimulate hair growth (Buhl et al, 1993).

K_{ATP} channels comprise a sulfonylurea receptor (SUR), amember of the ATP-binding cassette transporter, and an inward rectifer potassium channel (Aguilar-Bryan et al, 1998). SUR is considered to be a direct receptor for the K_{ATP} channel openers, which could function as a pump for ATP (Awqati, 1995; Schwanstecher et al, 1998). Thus far, however, no studies have been conducted to determine whether SUR is expressed in DPC. Concerning the activity of the K_{ATP} channel in cultured DPC, minoxidil failed to induce an increase in K^+

permeability, either in the K_{ATP} channel or in the Ca^{2+}-activated K^+ channels (Nakaya et al, 1994; Hamaoka et al, 1997), suggesting that minoxidilinduced hair growth may be due to other mechanisms that are independent of potassium channel opening.

Lachgar et al (1998) reported that minoxidil stimulates the production of growth factors such as vascular endothelial growth factor (VEGF) in cultured DPC, and that these growth factors might promote hair growth. The mechanism by which minoxidil increases the production of VEGF is not known, however.

Among the possible candidates for stimulators of VEGF production, adenosine seems to cause essential effects on a variety of cellular functions by triggering intracellular signal transduction and subsequent VEGF secretion in an autocrine or paracrine manner (Shryock and Belardinelli, 1997). With regard to possible sources of adenosine, ATP, which is secreted either through the ATP-binding cassette transporter or other currently unknown pathways (Awqati, 1995), is rapidly converted to adenosine by ecto-ATPase.

The aim of this study was to address whether minoxidil mediated by adenosine is capable of increasing $[Ca^{2+}]i$ levels

and VEGF production. Our fndings show that minoxidil caused an increase in $[Ca^{2+}]i$ levels and VEGF production in cultured DPC, and that these increases were inhibited by 8-sulfophenyl theophylline (8-SPT), a specific antagonist for adenosine A1/A2 receptors (A1R, A2R). This result suggests the presence of both adenosine receptors and a direct receptor for minoxidil, possibly SUR, in DPC. We were also able to confrm the gene expression of adenosine receptors and SUR in DPC by means of reverse transcription polymerase chain reaction (RT-PCR) analysis and subsequent sequence determination of the PCR products.

MATERIALS AND METHODS

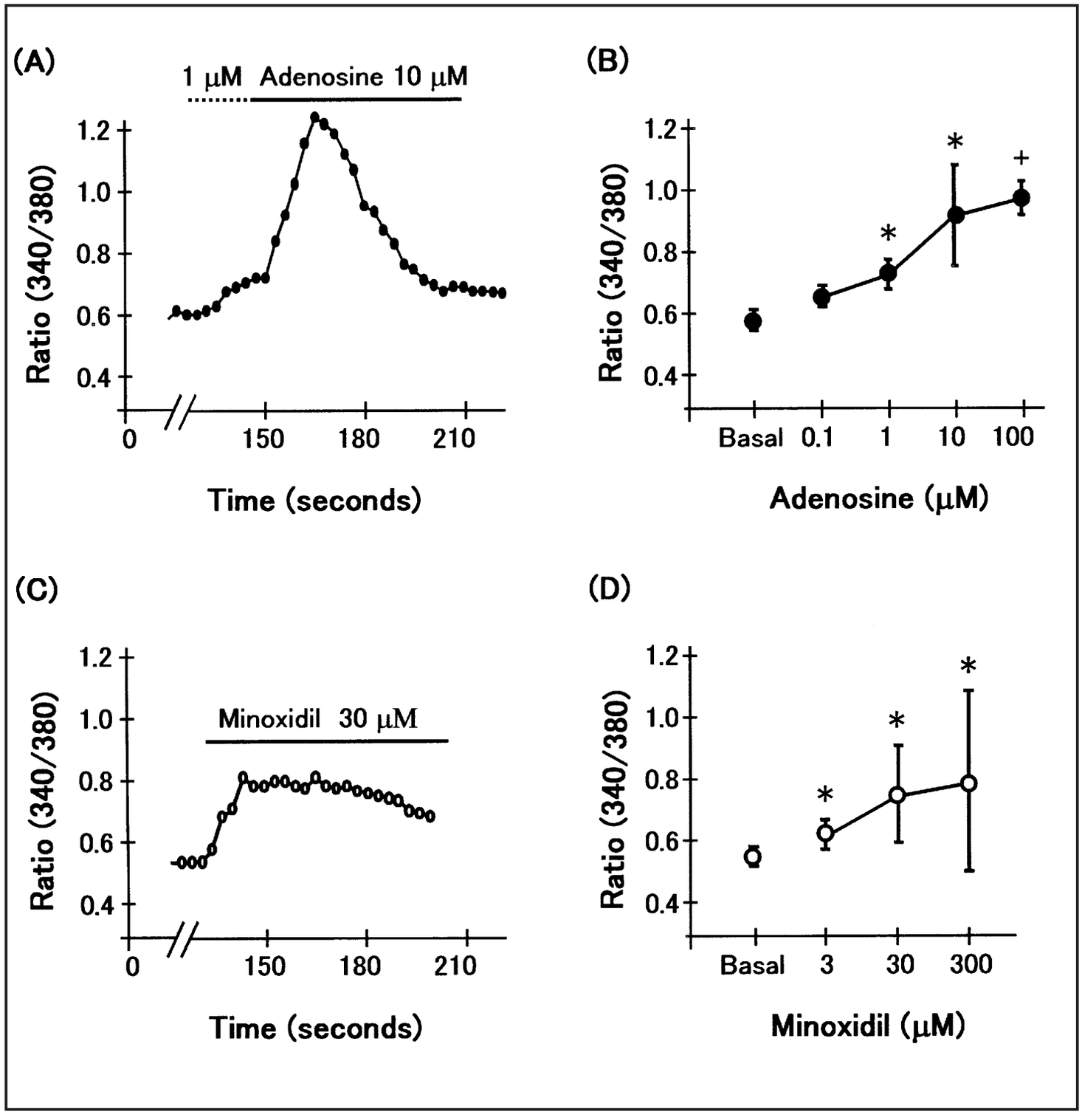
Cell culture DPC were cultured as described previously (Messenger, 1984). Dermal papillae were enucleated from excised hair follicles with the intact bulbous portion, and then cultured in Dulbecco's modifed Eagle's medium (DMEM) supplemented with 12% fetal bovine serum at 37°C in a humidifed atmosphere containing 5% CO_2/air. DPC were subcultured after they had grown out from the papillae and achieved subconfluence.

$[Ca^{2+}]i$ measurements Fura-2/acetoxymethyl ester (Wa-

Table I. Primers used in this study

Primer		Sequence
AR1	sense	5′-TGCTGAAGGCGTCGAGGTGT-3′
	antisense	5′-GCTTGCGGATTAGGTAGAAGAC-3′
A2AR	sense	5′-CATCATGGGCTCCTCGGT-3′
	antisense	5′-CTCTCCATCTGCTTCAGC-3′
A2BR	sense	5′-CAACTGCACAGAACCCTG-3′
	antisense	5′-GGCAGAGAAGATACCTGGAG-3′
SUR1	sense	5′-CTCCAACTACCTCAACTGGATGC-3′
	antisense	5′-TATGGGCAGGGTCCGAATGTGG-3′
SUR2A	sense	5′-CCACAAGAAGGGGAGATCAAGA-3′
	antisense	5′-CACTCCACTAAAATACCCTCAGAA-3′
SUR2B	sense	5′-CAAATGCACAGATGACAGACT-3′
	antisense	5′-TCTGCGCGAACAAAAGAAGC-3′

Database accession no.	Annealing temp.	Product size
GenBank X68485	60°C	765 bp
GenBank X68486	60°C	633 bp
GenBank X68487	55°C	479 bp
GenBank L78207	55°C	922 bp
DDBJ AF061317.1 (exon32 of SUR2) DDBJ AF061323.1 (SUR2A specific exon38)	55°C	662 bp
DDBJ AF061620.1 (exon35 of SUR2) DDBJ AF061324 (SUR2B specific exon38)	52°C	411 bp


(A)
1 μM Adenosine 10 μM
Ratio (340/380)
1.2
1.0
0.8
0.6
0.4
0
150
180
210
Time (seconds)
(B)
Ratio (340/380)
1.2
1.0
0.8
0.6
0.4
*
*
+
Basal
0.1
1
10
100
Adenosine (μM)
(C)
Minoxidil 30 μM
Ratio (340/380)
1.2
1.0
0.8
0.6
0.4
0
150
180
210
Time (seconds)
(D)
Ratio (340/380)
1.2
1.0
0.8
0.6
0.4
*
*
*
Basal
3
30
300
Minoxidil (μM)

ko Pharmaceuticals, Tokyo, Japan) was used for the measurement of $[Ca^{2+}]i$ concentration. Cells were cultured on 13 mm cover glasses (Matsuura Industry, Tokyo, Japan) and washed with modified normal Tyrode's solution, which contained 140 mM NaCl, 4.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM 3-morpholinopropanesulfonic acid, and 10 mM glucose (pH 7.2–7.4). Fura-2/acetoxymethyl ester (4 μM) was added and the mixture was incubated for 30 min at 37°C. The cover glass was then transferred to the circulated temperature-

Figure 1. Changes in $[Ca^{2+}]i$ levels caused by adenosine and minoxidil. The test solution containing adenosine (10 μM) caused a significant increase in $[Ca^{2+}]i$ levels in cultured DPC, as evidenced by the appearance of an initial peak (A). The test solution containing minoxidil (30 μM) alone also caused a significant increase in $[Ca^{2+}]i$ levels (C), at a slower rate than that with adenosine, followed by a steady increase over time. Concentration-dependent elevations of $[Ca^{2+}]i$ are shown in (B) and (D), respectively. The increase was significant at 100 μM in adenosine and at 300 μM in minoxidil ($n > 7$ at each point). *, +, $p < 0.05$ and $p < 0.01$ vs control, respectively.

controlled chamber of the analysis system, an ARGUS-50 (Hamamatsu Photonics, Hamamatsu, Japan), and the test solution was perfused. Any emitted fluorescence in DPC on the cover slips was measured using a fluorescence spectromicroscope (excitation, 340/380 nm; emission, 510 nm). The results were expressed as the ratio of the fluorescence recorded at 340 nm and380 nm. Changes in this ratio were detected when minoxidil [in this study, minoxidil sulfate was used as the active form of minoxidil, and was synthesized according to the method described by Newgreen et al (1990)] or adenosine (Wako) was added to the test solution alone or together with one of the following: the antagonist for A1R/A2R, 8-SPT (Sigma-Aldrich Chemie, Steinheim, Germany); a specific antagonist for A1R, 8-cyclopentyl-1,3-dipropylxanthine (CPX) (Sigma); or a specific antagonist for A2R, 3,7-dimethyl-1-propargyl-xanthine (DMPX)(Sigma).

Secretion of VEGF Secreted VEGF from cultured DPC was determined using a kit for the sandwich enzyme-linked immunosorbent assay (ELISA) of human VEGF (Genzyme/TECHNE, Minneapolis, MN). After reaching subconfluence, DPC were cultured in a 35 mm dish with 1% fetal bovine serum for 24 h. Adenosine or minoxidil was added to 90%

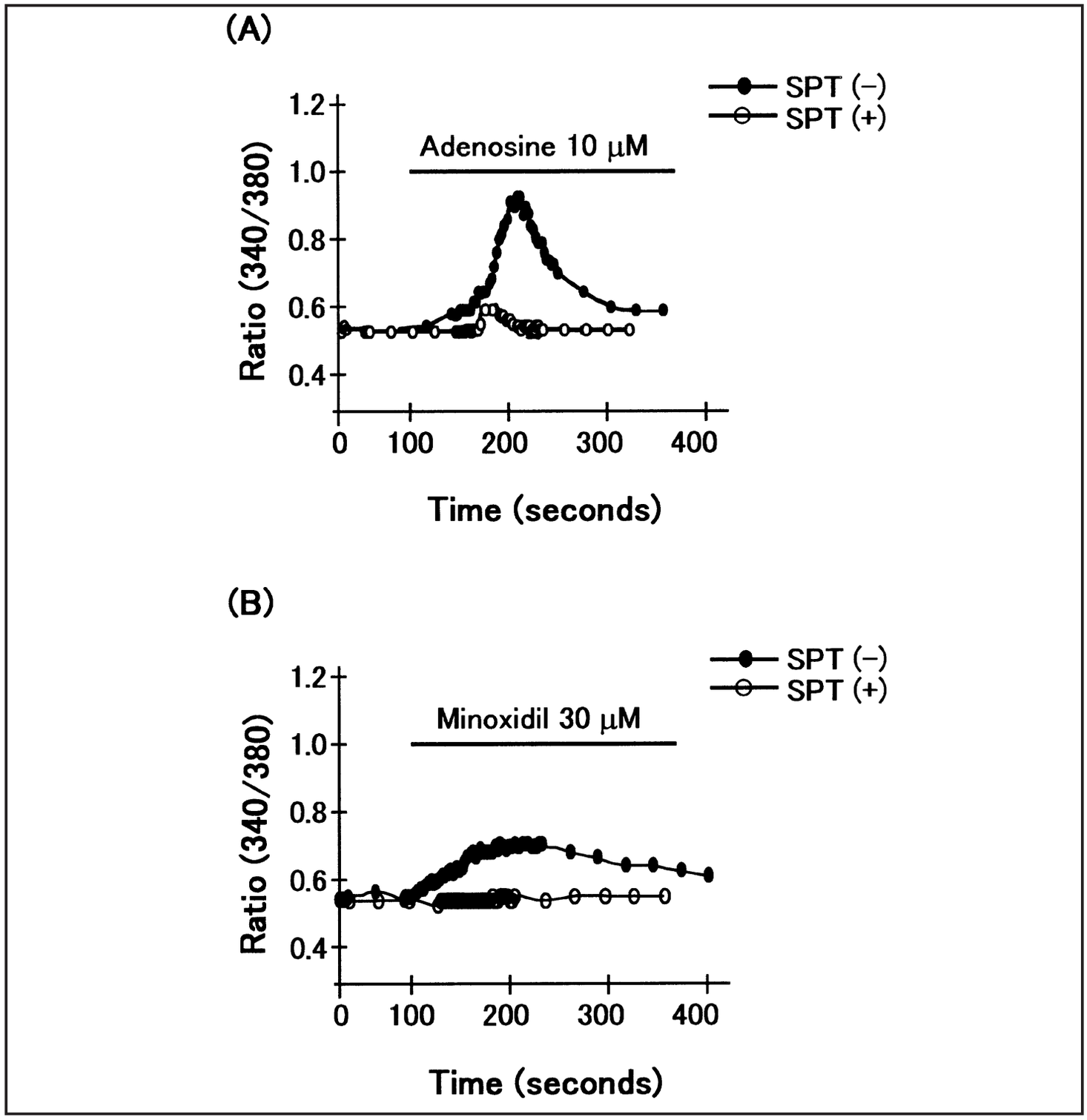
confluent cultures of DPC alone or together with antagonists for adenosine receptors. Culture media were then collected for measurements. Because adenosine and minoxidil are not stable, these compounds (10 µl of 10^{-5} M solution) were added to the culture media at 6 h intervals. After 24 h, the culture media were collected in order to measure VEGF.

A monoclonal antibody specific for VEGF was precoated onto a microplate. The collected supernates were pipetted into the wells, where VEGF in the sample was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody, which is specific for VEGF, was added to the wells. Following a wash to remove any unbound antibody–enzyme reagent, a substrate solution was added to the wells, and the colors developed were proportional to the amount of VEGF bound in the initial step. Color development was stopped and the intensity of the color was measured.

RT-PCR and sequence analysis After reaching subconfluence in DMEM medium, DPC were washed with phosphate-buffered saline (PBS). Total RNA was extracted from the cells using the acid guanidinium–phenol–chloroform method (Chomczynski and Sacchi, 1987). To determine

expression of SURs and adenosine receptors in DPC, the reverse transcription of 5 mg of total RNA was performed using the Superscript Preamplifcation System for First Strand cDNA Synthesis according to the manufacturer's instructions (Life Technologies, Rockville, MD). The PCR was carried out according to a previously described method (Rappolee et al, 1988). In brief, the reaction was performed in 50 µl of reaction mixture, containing 2 µl of reverse transcription mixture prepared by the preamplifca-

Figure 2. Effects of a specific antagonist for adenosine receptors on the minoxidil-induced increase in $[Ca^{2+}]i$ levels. The effect of the antagonist for A1R/A2R, 8-SPT, was tested on the adenosine-induced increase in $[Ca^{2+}]i$ levels (A). In the presence of 8-SPT (*open circles*), the increase in $[Ca^{2+}]i$ levels by adenosine (*closed circles*) was blocked (n = 8). The effect of 8-SPT was tested on the minoxidil-induced increase in $[Ca^{2+}]i$ levels (B). In the presence of 8-SPT (*open circles*), minoxidil (n = 10) did not cause an increase in $[Ca^{2+}]i$ levels, compared with the increase caused by the test solution containing minoxidil alone (*closed circles*).


(A)
SPT (–)
SPT (+)
Adenosine 10 μM
Ratio (340/380)
1.2
1.0
0.8
0.6
0.4
0
100
200
300
400
Time (seconds)
(B)
SPT (–)
SPT (+)
Minoxidil 30 μM
Ratio (340/380)
1.2
1.0
0.8
0.6
0.4
0
100
200
300
400
Time (seconds)

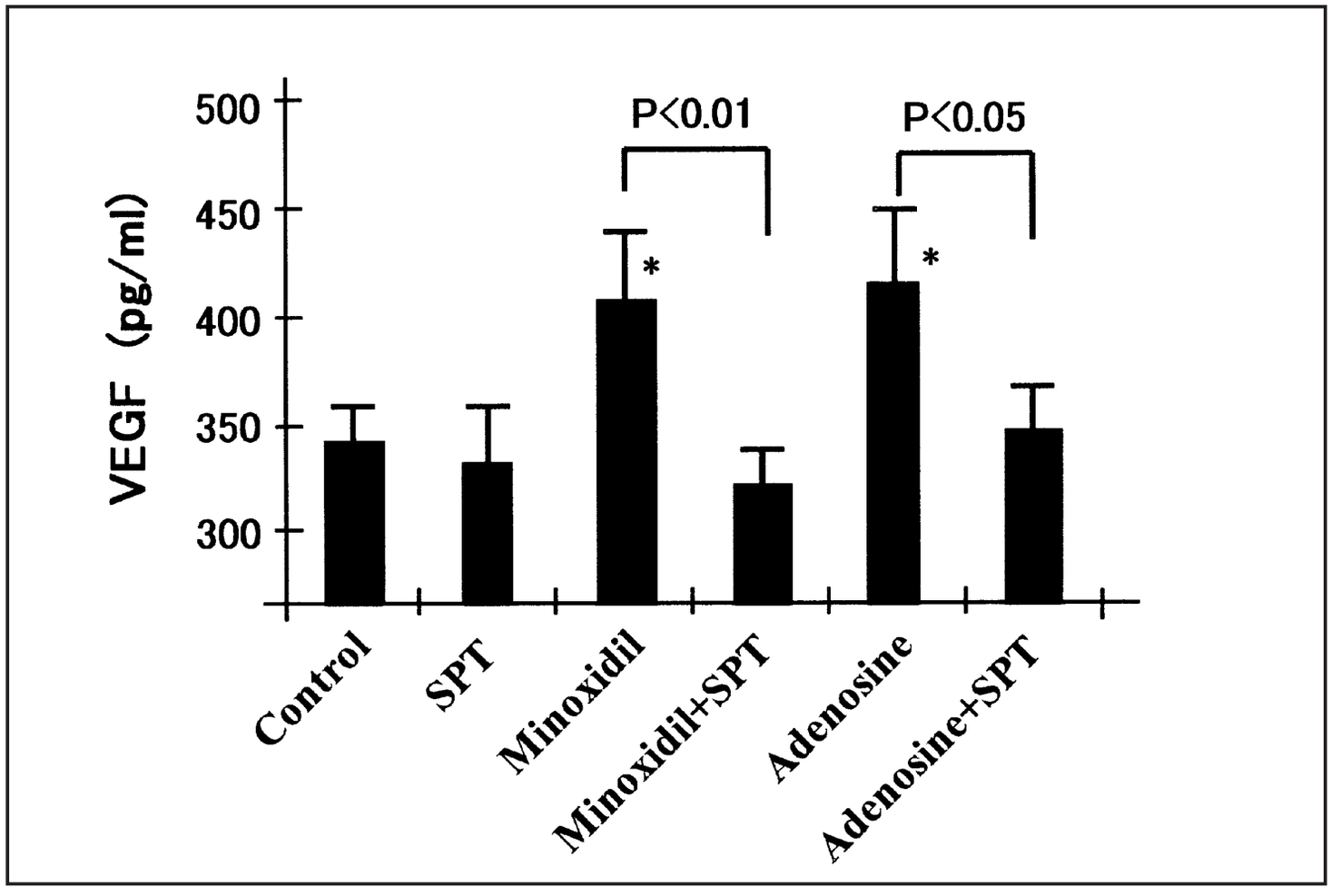


Figure 3. Production of VEGF by adenosine or minoxidil in cultured DPC. The addition of 10 μM adenosine (n = 8) or 30 μM minoxidil (n = 8) for 24 h increased the production of VEGF. These increases were significantly suppressed in the presence of 1 μM 8-SPT, an A1R/A2R antagonist. 1 μM SPT alone did not cause a change in VEGF production in cultured DPC. *$p < 0.05$ vs control.

tion system described above, 1 ml of each gene-specific primer (10 µM), and 0.5 µl of 5 units per µl "long and accurate" (LA) taq DNA polymerase (Takara Biotechnology, Kyoto, Japan). After an initial denaturation step for 1 min at 94°C, the DNA was amplifed for 30 cycles at 94°C for 30 s, various annealing temperatures for 30 s, and at 72°C for 2 min on a DNA cycler, followed by electrophoresis. Six pairs of specific oligonucleotide primers for each of the receptors were used, as shown in Table I, and were synthesized by Gibco BRL (Tokyo, Japan). Specific primers for human GAPDH (5′-CCACCCATGGCAAATTCCATGGCA-3′, sense, and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′, antisense) were purchased from Clontech (Tokyo, Japan). The amplifed DNA fragments were subcloned into a pT-7Blue cloning vector (TaKaRa Shuzo, Shiga, Japan) and sequenced by means of a BigDye terminator cycle sequencing kit in an ABI 310 genetic analyzer (ABI PRISM 310, PE Applied Biosystems).

Statistical analysis Student's t test was used to determine the significance of differences in findings before and after the application of adenosine receptor antagonist. p-values of less than 0.05 were considered significant.

RESULTS

Changes in $[Ca^{2+}]i$ levels by minoxidil and adenosine Based on the fact that intracellular calcium is a ubiquitous second messenger, which regulates a wide range of cellular processes associated with a variety of fundamental cellular functions (Toescu, 1995), including VEGF gene expression (Mukhopadhyay and Akbarali, 1996), we first examined the effect of adenosine and minoxidil on $[Ca^{2+}]i$ levels in cultured DPC. As shown in Fig 1(A), adenosine (10 μM) initially increased the levels of $[Ca^{2+}]i$ (ratio of 340/380 nm) in cultured DPC, followed by a small sustained elevation of $[Ca^{2+}]i$. The relation between the dose of adenosine and the elevation of the $[Ca^{2+}]i$ peak is shown in Fig 1(B).

Minoxidil (30 μM) caused a gradual increase in $[Ca^{2+}]i$ levels over time in cultured DPC (Fig 1C), although the rate of elevation was slow. The dose-dependent increase in $[Ca^{2+}]i$ levels by minoxidil is shown in Fig 1(D). In the absence of extracellular Ca^{2+}, neither minoxidil nor adenosine caused an increase $[Ca^{2+}]i$ levels (data not shown).

In order to determine whether the minoxidil-induced increase in $[Ca^{2+}]i$ levels is mediated by adenosine, the effect

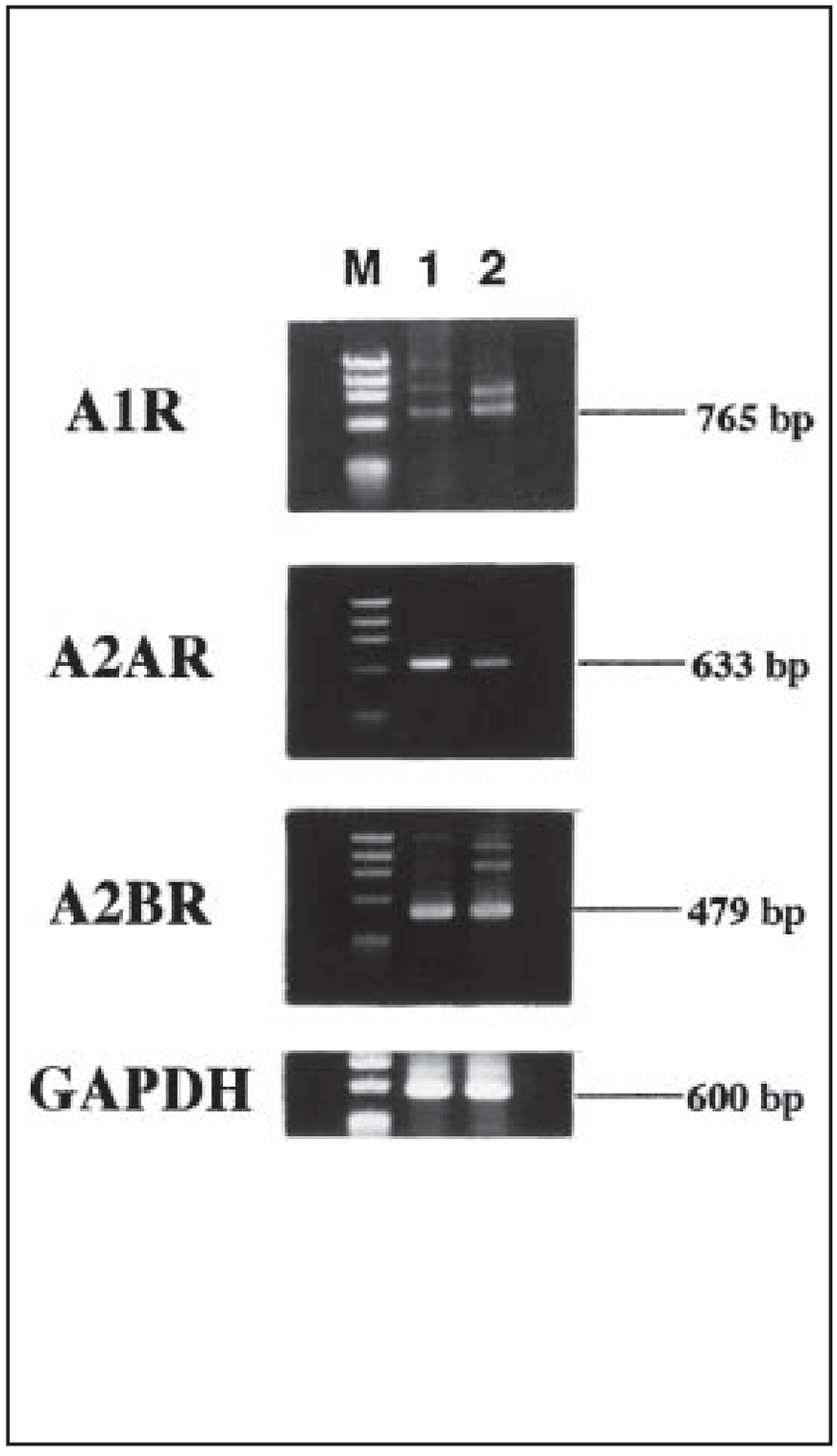


Figure 4. RT-PCR analyses confirmed the expression of adenosine receptors in DPC. cDNAs from DPC (*lane 2 s*) were amplified with primers specific for A1R, A2AR, and A2BR. Lane 1 s shows the PCR product of human vascular endothelial cells, which were used as a positive control. GAPDH mRNA was used as a quantitative RNA control. The sizes expected for the specific PCR products are shown on the right. M, marker.

of an A1R/A2R antagonist, 8-SPT, was examined (Fig 2). In the presence of 1 μM 8-SPT alone, the fluorescence ratio of 340/380 nm was not altered, although it was increased at higher concentrations of 8-SPT.

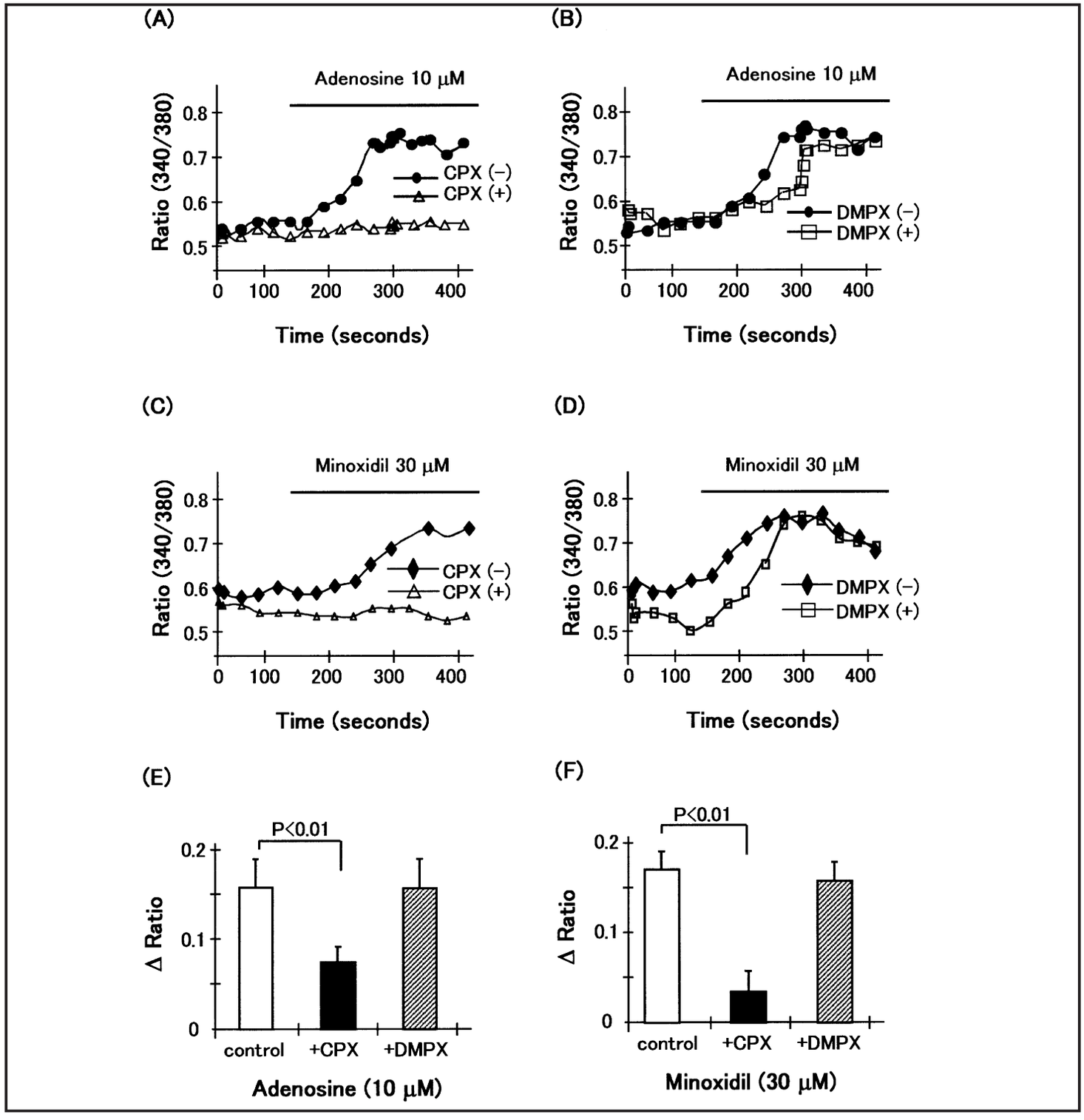
Therefore, we used 1 μM 8-SPT in this experiment. The increase in $[Ca^{2+}]i$ levels, which was induced by the presence of 10 μM adenosine or 30 μM minoxidil, was almost completely blocked by 1 μM 8-SPT (Fig 2A, B, *open circles*).

Changes in VEGF secretion caused by minoxidil and adenosine Figure 3 shows the secretion of VEGF from cultured DPC by minoxidil or adenosine. Minoxidil, at a concentration of 30 μM, and adenosine, at a concentration of 10 μM, signifcantly increased the production of VEGF (both $p < 0.05$), and these increases were suppressed in the presence of 1 μM 8-SPT (both $p < 0.05$). 1 μM 8-SPT alone had no effect on the production of VEGF.

Gene expression of adenosine receptors in DPC The increases in $[Ca^{2+}]i$ levels were inhibited by the A1R/A2R antagonist, suggesting the presence of adenosine receptors in DPC. We therefore examined whether the genes encoding adenosine receptors were expressed in DPC, and, if so, which subtype is expressed. RT-PCR reactions were performed using

specific primers for the A1R, A2AR, and A2BR, followed by agarose gel electrophoreses of the products. The expected sizes of the specific PCR products were 765 bp for A1R, 633 bp for A2AR, and 479 bp for A2BR (Fig 4). DPC showed bands corresponding to the expected size of all three of these subtypes of adenosine receptors. Sequence analyses of these products were performed after subcloning them into the pT7Blue cloning vector. All of the sequences were identical with those found in the DDBJ database (data not shown).

Effect of subtype-specific adenosine receptor antagonist on minoxidil-induced increase in $[Ca^{2+}]i$ levels and VEGF production Based on the finding that gene expression of all of A1R, A2AR, and A2BR were detected in DPC, we focused attention on which of these receptors contributes to the minoxidil-induced increase in $[Ca^{2+}]i$ levels and VEGF production. The receptor subtypes involved were characterized using the selective receptor antagonists CPX and DMPX, selective antagonists for A1R and A2R, respectively. The increase in $[Ca^{2+}]i$ levels, as induced by 10 μM adenosine (Fig 5A, B, *closed circles*) was almost completely blocked by 5 nM CPX (Fig 5A, *open triangles*), but not by 3 μM DMPX (Fig 5B, *open squares*). Similar results were obtained


(A)
Adenosine 10 μM
Ratio (340/380)
0.8
0.7
0.6
0.5
CPX (−)
CPX (+)
0
100
200
300
400
Time (seconds)
(B)
Adenosine 10 μM
Ratio (340/380)
DMPX (−)
DMPX (+)
Time (seconds)
(C)
Minoxidil 30 μM
Ratio (340/380)
CPX (−)
CPX (+)
Time (seconds)
(D)
Minoxidil 30 μM
Ratio (340/380)
DMPX (−)
DMPX (+)
Time (seconds)
(E)
P<0.01
Δ Ratio
0.2
0.1
0
control
+CPX
+DMPX
Adenosine (10 μM)
(F)
P<0.01
Δ Ratio
0.2
0.1
0
control
+CPX
+DMPX
Minoxidil (30 μM)

when minoxidil was used in place of adenosine (Fig 5C, open triangles, and Fig 5D, open squares). To exclude the possibility of direct involvement of the adenosine receptor antagonist in the changes in $[Ca^{2+}]i$ levels, either 5 nM CPX or 3 μM DMPX alone was added to the DPC culture. In the pres-

Figure 5. Effects of subtype-selective antagonists for adenosine receptors on the minoxidil-induced increase in $[Ca^{2+}]i$ levels. The increases caused by either adenosine (n = 8) or minoxidil (n = 8) were completely inhibited when 5 nM CPX, a selective antagonist for A1R, was added to the test solution (*open triangles, A and C*), but this was not the case when 3 μM DMPX, a selective antagonist for A2R, was added (*open squares, B and D*). (A) The test solution contained adenosine in the presence (*open triangles*) or absence (*closed circles*) of CPX; (B) adenosine in the presence (*open squares*) or absence (*closed circles*) of DMPX; (C) minoxidil in the presence (*open triangles*) or absence (*closed diamonds*) of CPX; (D) minoxidil in the presence (*open squares*) or absence (*closed diamonds*) of DMPX. Statistical analysis showed a significant inhibitory effect of CPX on both the adenosine-induced (E) and minoxidil-induced (F) increase in $[Ca^{2+}]i$ levels.

ence of either of these, the fluorescence ratio of 340/380 nm was not altered (data not shown). To summarize, a signifcant inhibition in the increase in $[Ca^{2+}]i$ levels caused by adenosine (Fig 5E) or minoxidil (Fig 5F) was observed in the case of CPX (both $p < 0.01$), whereas no change was observed when DMPX was used.

We also tested the effects of CPX and DMPX on minoxidil-induced secretion of VEGF. In contrast, the increase in VEGF production induced by 10 μM adenosine or 30 μM minoxidil was inhibited by both 5 nM CPX (both $p < 0.05$) and 3 μM DMPX (both $p < 0.05$) (Fig 6). Neither 5 nM CPX nor 3 μM DMPX altered the production of VEGF.

Gene expression of SUR2B in DPC Based on the data presented above, we assume that the effect of minoxidil is mediated by adenosine in cultured DPC, which suggests the endogenous production of adenosine or a precursor thereof in response to minoxidil. Given that SUR might function as a pump for ATP (Awqati, 1995), the expression of SUR, a target receptor for K_{ATP} channel openers, was investigated in DPC. RT-PCR was performed using specific primers for SUR1, SUR2A, and SUR2B, followed by agarose gel electrophoreses of the products. Among these three subtypes, only the SUR2B

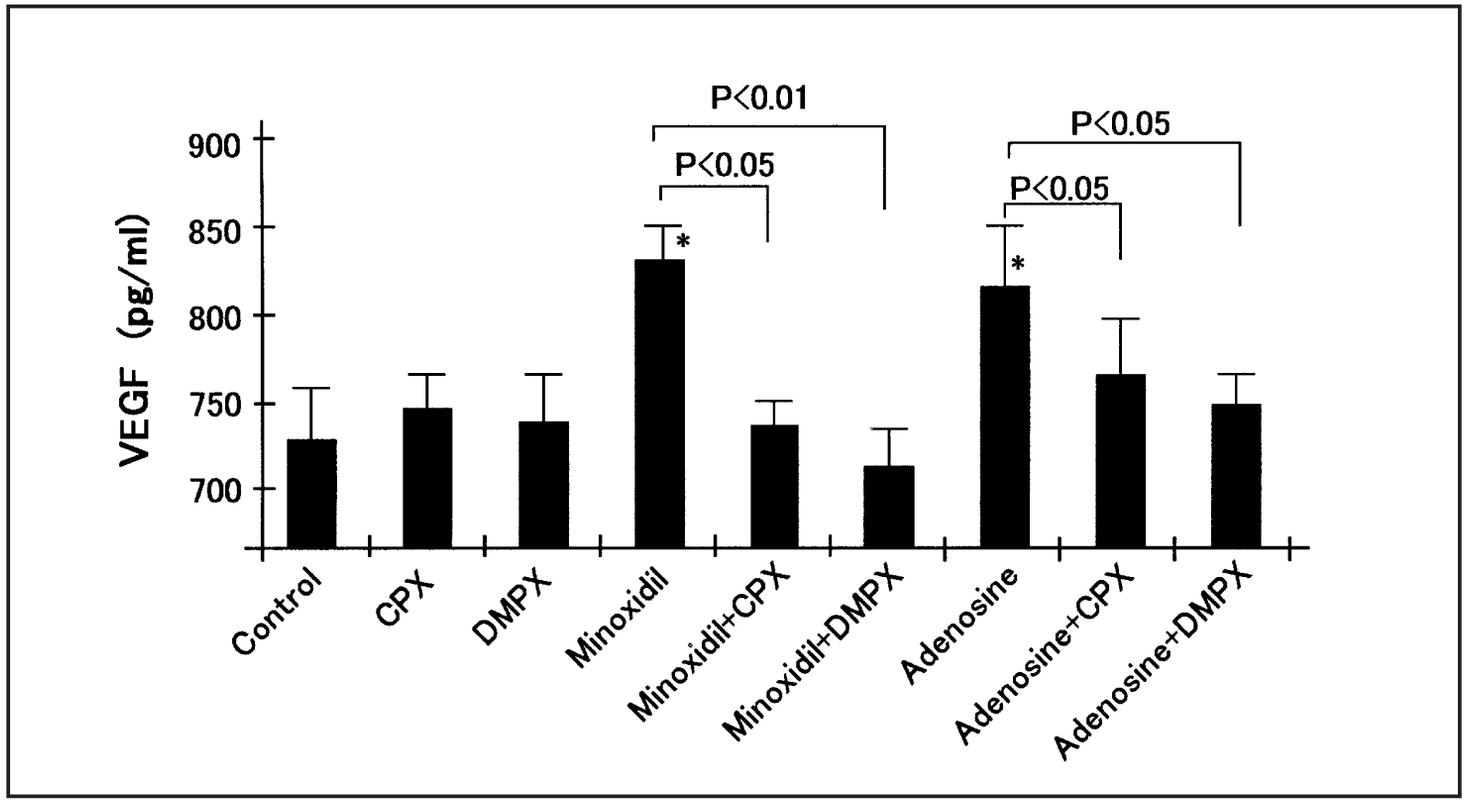


Figure 6. Effects of subtype-selective antagonists for adenosine receptors on the minoxidil-induced VEGF production. The increase in VEGF production caused by either adenosine (n = 5) or minoxidil (n = 5) was inhibited when 5 nM CPX or 3 μM DMPX was added to the test solution for 24 h of incubation. VEGF production induced by 10 μM adenosine or 30 μM minoxidil was inhibited by 5 nM CPX and 3 mM DMPX, respectively. Neither 5 nM CPX nor 3 μM DMPX had any effect on VEGF production in cultured DPC. *p <0.05 vs control.

product was detected at the expected size of 411 bp (Fig 7). Subsequent sequence analysis revealed that the sequence was identical to that submitted to the DDBJ database (data not shown).

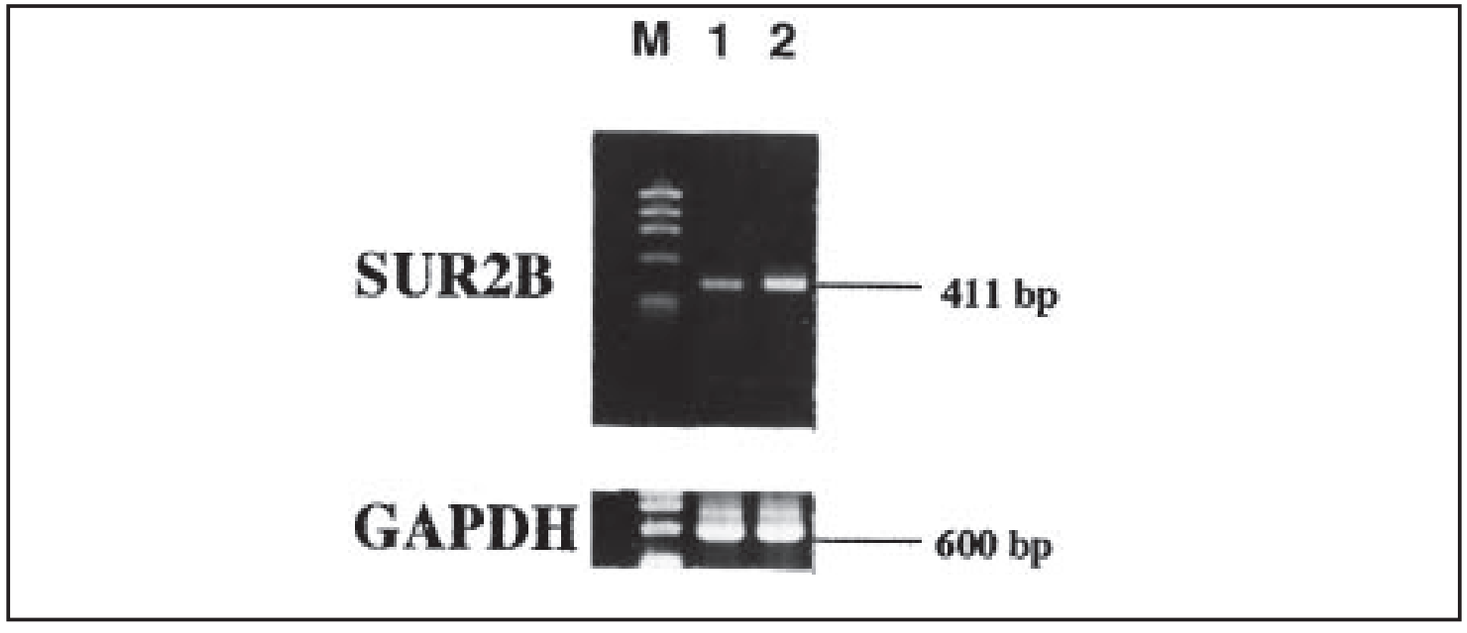


Figure 7. RT-PCR analysis confirmed the expression of SUR2B in DPC. cDNAs from DPC (*lane 2*) were amplified with primers specific for SUR2B. The PCR product of human aortic smooth muscle cell cDNA (*lane 1*) was used as a positive control and GAPDH mRNA was used as a quantitative RNA control. The size expected for the specific PCR product is shown on the right. M, marker.

DISCUSSION

The elevation of $[Ca^{2+}]i$ and the production of VEGF are known to stimulate cell proliferation. This study showed that minoxidil increased both $[Ca^{2+}]i$ levels and VEGF production in cultured DPC in a manner similar to that of adenosine, and these increases were inhibited by an adenosine receptor antagonist. Thus, we hypothesize that the stimulation of DPC by minoxidil activated an adenosine/adenosine receptor signal transduction pathway (Fig 8). In support of this hypothesis, the presence of adenosine receptors in DPC was investigated, revealing the presence of all of the A1R, A2AR, and A2BR subtypes.

VEGF not only is a potent stimulator of vasodilation, microvascular hyperpermeability, and angiogenesis, but also serves as a multifunctional growth factor for a variety of cells. Lachgar et al (1996) reported that DPC express VEGF, and that VEGF acts on DPC as an autocrine growth factor. They (Lachgar et al, 1998) also found that the production of VEGF is promoted by minoxidil in cultured DPC, and concluded that minoxidil promotes hair growth via the induced VEGF. The molecular mechanisms governing VEGF production by

minoxidil have not yet been clearly elucidated, however.

Adenosine stimulates VEGF production through the activation of cell surface adenosine receptors (Hashimoto et al, 1994; Takagi et al, 1996). This study has shown that minoxidil, like adenosine, has a positive effect on promoting $[Ca^{2+}]i$ and VEGF production, and that these effects are blocked by specific adenosine receptor antagonists, indicating that minoxidil-induced hair growth is mediated by adenosine. The gene expression of adenosine receptors in DPC confirmed the endogenous production of VEGF. The structure of adenosine receptors governs the various facets of subtype activity (Olah and Stiles, 2000). A functional subtype determination experiment in our study demonstrated that the receptors involved in the effect of increasing $[Ca^{2+}]i$ levels belong to the A1R subtype, and that, on the other hand, VEGF production could be activated via both A1R and A2R within the same DPC. These results indicate that DPC themselves possess multiple adenosine-dependent signaling pathways.

The opening of K_{ATP} channels has been considered to be a common mechanism for the action of minoxidil and a set of potassium channel openers (Buhl et al, 1993). Several investigators have described a benefcial effect of the K_{ATP} channel

on cardioprotective vasorelaxation associated with adenosine receptors (Kato et al, 2000; Roscoe et al, 2000). There are three possibilities regarding this process: (i) K_{ATP} channels could activate adenosine receptors directly, although no evidence is currently available to suggest that a binding site for K_{ATP} channel openers exists in adenosine receptors — thus far, SUR is considered to be a receptor for minoxidil, and to have a specific binding site for the K_{ATP} channel opener; (ii) K_{ATP} channels couple to adenosine receptors via mediators, such as G proteins (Kirsch et al, 1990). (iii) K_{ATP} channel openers act not only to open an inward rectifier potassium channel but also to promote the secretion of ATP either through SUR or via an unknown pathway (Awqati, 1995; Kitakaze et al, 1996). ATP is rapidly converted to adenosine by ecto-ATPase. Our results strongly indicate the possibility that a contribution by SUR has an effect on the minoxidil-activated adenosine signal transduction pathway.

Three subtypes of SURs have been recognized to date: pancreatic type (SUR1), the inhibition of which facilitates insulin secretion; cardiac type (SUR2A), which provides myocardial protection; and vascular smooth muscle type (SUR2B), which plays a role in vasorelaxation (Yokoshiki et al, 1998).

Differential sensitivity to sulfonylureas or dizaoxide has been shown among these three isoforms (Aguilar-Bryan et al, 1998). This study has shown that SUR2B, but not SUR1 or SUR2A, is present in DPC, which is consistent with the report that minoxidil has a specific affinity for SUR2B (Schwanstecher et al, 1998). The expression of SUR2B in DPC provides a possible binding site to minoxidil and a potential mediator for adenosine production.

We postulated that the adenosine/VEGF pathway in cultured DPC might play an important role in hair growth. Our study was performed in cultured cells, however, which might be a limitation of this study. A recent in situ study (Yano et al, 2001) showed that upregulation of VEGF mRNA was found only in the outer root sheath keratinocytes, but not in DPC, of the anagen murine hair follicle. The angiogenic activity of the rat vibrissa hair follicle associated with the epithelial hair bulb (Stenn et al, 1988) might be partly due to this upregulation. A study by Lachgar et al (1998) and this study, however, showed definitively increased VEGF expression in cultured DPC by treatment with minoxidil. In addition, Kozlowska et al (1998) showed the immunohistochemical expression of VEGF in DPC. The discrepancy between in vitro

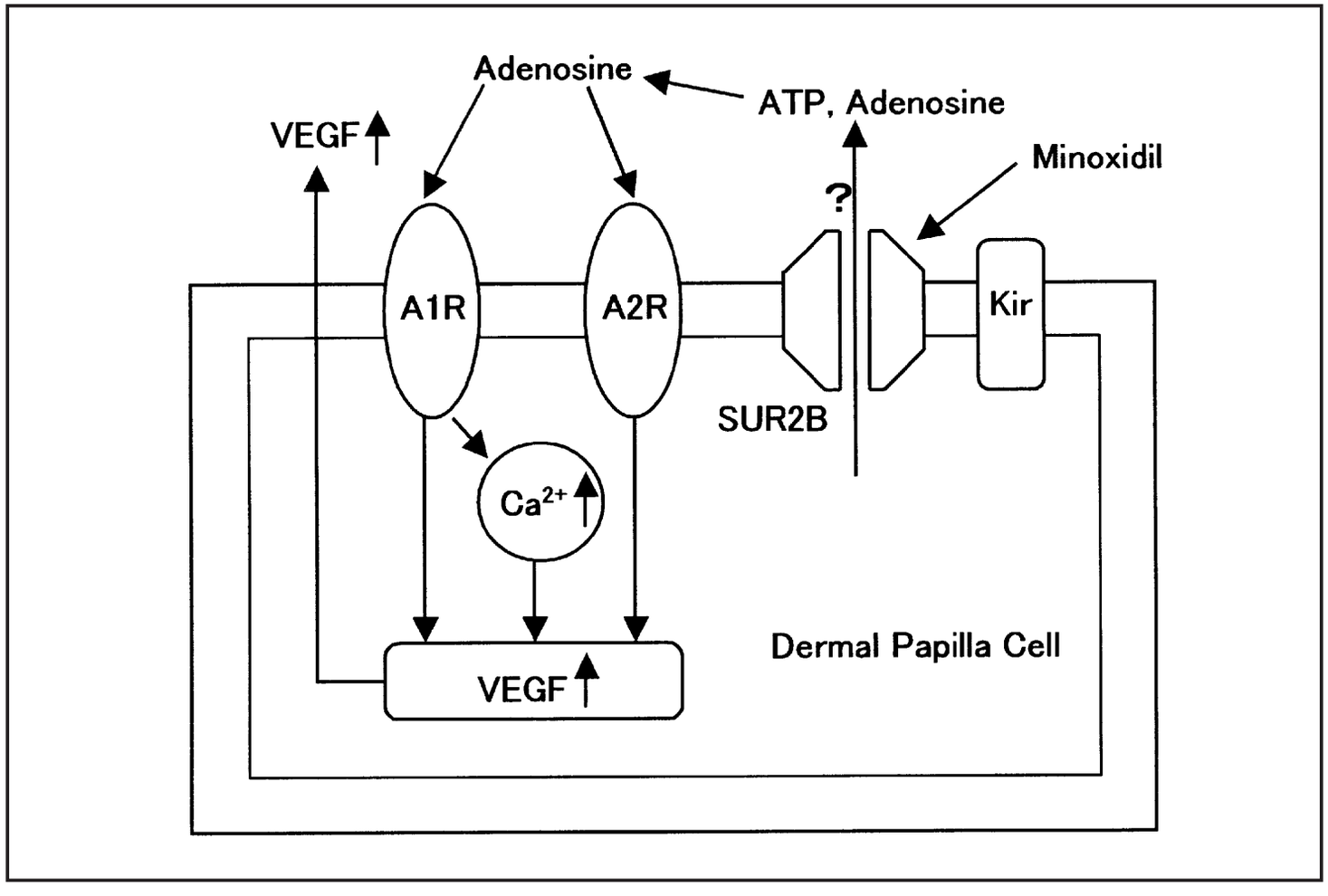


Figure 8. Proposed signaling pathway mediating minoxidil action in cultured DPC. The proposed pathway links minoxidil-induced VEGF upregulation to adenosine, and adenosine receptors mediate the increase in $[Ca^{2+}]i$ levels and VEGF production in cultured DPC. SUR is proposed to be a target receptor for minoxidil and a source of ATP and adenosine production, respectively.

and in vivo results has also been observed in the apoptosis of DPC. It is notable that DPC undergo apoptosis in culture (Ferraris et al, 1997) but appear not to during embryogenesis (Wessels and Roessner, 1965) and throughout the hair cycle in adults (Couchman, 1993; Nutbrown and Randall, 1995), suggesting that the normal quiescent state of DPC in vivo may confer protection from induction of cell death. As natural DPC exist in a complex epithelial–mesenchymal microenvironment, some characteristics in vitro may be suppressed in order to keep the physiologic balance. The inherent abilities of DPC might be released in vivo under the influence of special endogenous pathologic factors or exogenous stimulation. Given that adenosine-induced upregulation of VEGF expression is found in various cell types both in vivo (Martin et al, 1998) and in vitro (Gu et al, 1999), a similar mechanism on cultured DPC by minoxidil is expected to found in vivo under the influence of stimulation with topical minoxidil.

Herein, we propose a mechanism for minoxidil-induced VEGF production in cultured DPC, and conclude that an adenosine-mediated signal transduction pathway contributes to minoxidil-induced hair growth. The gene expression of a number of receptors such as SUR2B, along with that of

adenosine receptors in DPC, emphasizes the important role of DPC in hair growth.

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Reprint requests to: Prof. Seiji Arase, Department of Dermatology, The University of Tokushima, School of Medicine, 3–18, Kuramoto-cho, Tokushima, Japan 770-8503. Email: sarase@clin.med.tokushima-u.ac.jp

Abbreviations: A1 (2A, 2B) R, adenosine A1 (A2A, A2B) receptor; CPX, 8-cyclopentyl-1,3-dipropylxanthine; DMPX, 3,7-dimethyl-1-propargyl-xanthine; DPC, dermal papilla cell; K_{ATP} channel, ATP-sensitive potassium channel; 8-SPT, 8-sulfophenyl theophylline; SUR, sulfonylurea receptor; VEGF, vascular endothelial growth factor.

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ORIGINAL CONTRIBUTIONS

Investigation of the topical application of procyanidin oligomers from apples to identify their potential use as a hair-growing agent

Tomoya Takahashi,[1] Ayako Kamimura,[1] Masayori Kagoura,[2]
Masahiko Toyoda[3] & Masaaki Morohashi[3]

[1] Tsukuba Research Laboratories
Kyowa Hakko Kogyo Co., Ibaraki, Japan

[2] Kagoura Dermatologic Clinic, Toyama, Japan

[3] Department of Dermatology
Toyama Medical and Pharmaceutical University, Toyama, Japan

Summary

Background Procyanidins are a family of condensed tannins, which have been shown to possess hair-growing activity in both the in vitro and in vivo murine models.

Aims We report a 12-month clinical study aimed at treating male pattern baldness by external application of 0.7% apple procyanidin oligomers.

Patients/methods A double-blind clinical test involving a total of 43 subjects was performed. Twenty-one men in the procyanidin group and 22 men in the placebo control group were subjected to analysis. In the first 6 months, we compared the procyanidin and the placebo groups to assess the medicinal effects of procyanidin oligomers. The application time of the procyanidin group was subsequently extended to 12 months, and the time course of its remedial value was examined.

Results The increase in total number of hairs in a designated scalp area of the procyanidin group subjects after the 6-month trial was significantly greater than that of the placebo control group subjects (procyanidin, 3.3±13.0 (mean±SD)/0.50 cm^2; placebo, –3.6±8.1/0.50 cm^2; $P<0.001$, two-sample t-test). Time course-dependent improvement in hair density was

observed in the procyanidin subjects. No adverse side effects were observed in any of the subjects. Procyanidin therapy thus shows potential hair-growing activity.
Keywords: external application, male pattern baldness, Malus pumila, procyanidin oligomers, scalp

Introduction

Proanthocyanidins are a species of polyphenol known to possess a variety of physiological activities, including radical scavenging activity,[1] antioxidative properties,[2] antifungal effects,[3] antiallergic activity all in vitro,[4] and antihypertensive activity in vivo,[5] and have been used as a treatment for capillary stabilization.[6] We have reported that procyanidin oligomers such as procyanidin B-2 (Fig. 1) possess growth-promoting activity in murine hair epithelial cells at a very high rate of 300% relative to controls, and have also demonstrated that procyanidin oligomers stimulate anagen induction in the in vivo murine model.[7] We isolated highly purified procyanidin oligomers, in particular procyanidin B-1, procyanidin B-2, and procyanidin C-1 from apples on an industrial scale[8] and subjected them to a series of toxicological studies. Our results confirmed the safety of topical application

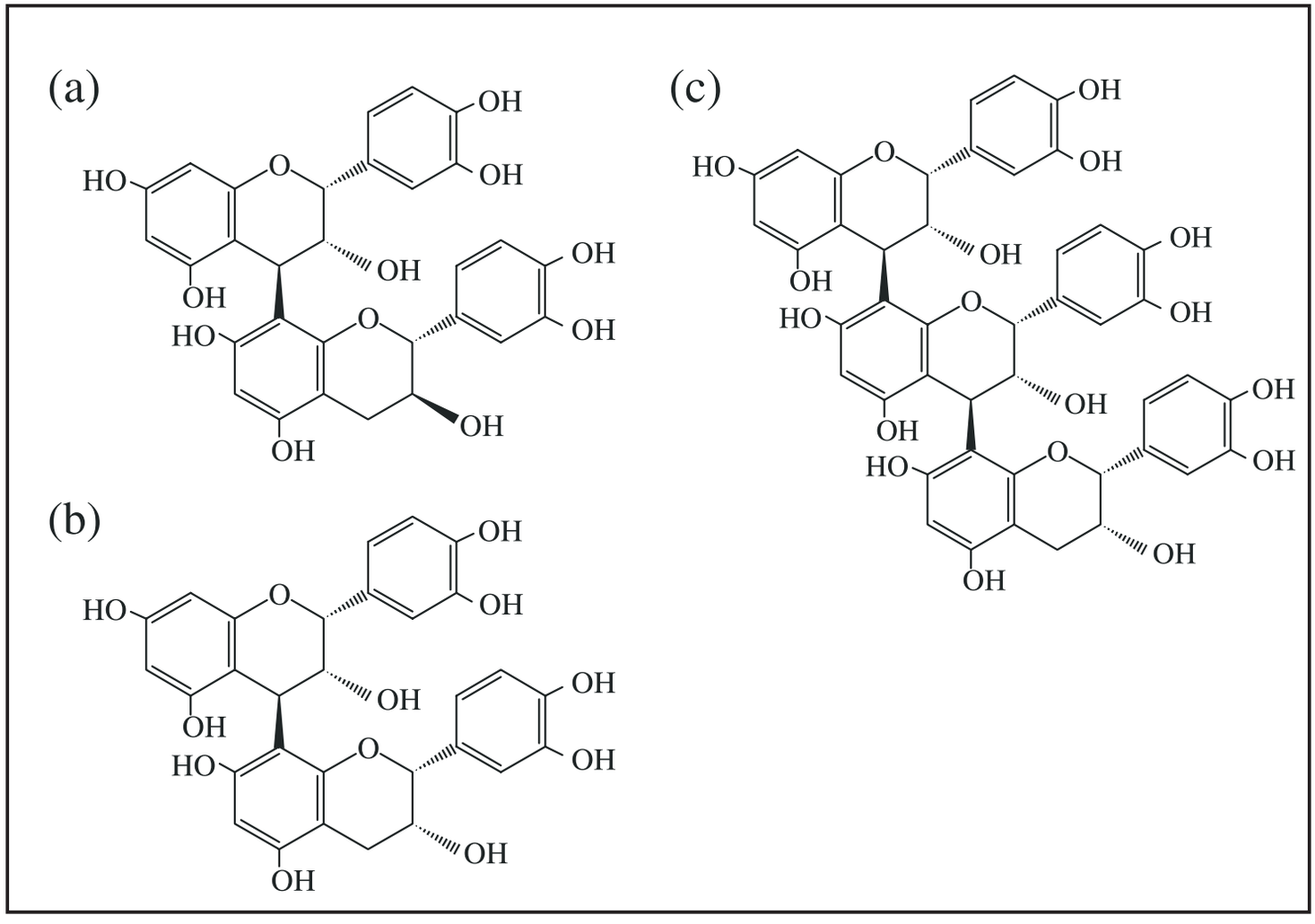


Figure 1 Structures of procyanidin oligomers. (a), Procyanidin B-1 [epicatechin-(4β(→)8)-catechin]; (b), procyanidin B-2 [epicatechin-(4β(→)8)-epicatechin]; (c), procyanidin C-1 [epicatechin-(4β(→)8)-epicatechin-(4β(→)8)-epicatechin].

of procyanidin oligomers to human skin. We report here the results of a volunteer test focusing on the topical application of procyanidin oligomers of high purity to test whether or not these procyanidin oligomers exhibited a curative effect on male pattern baldness or a delaying effect on the development of male pattern baldness; thus, changes in hair density was assessed.

Materials and methods

Subjects

Volunteer subjects (27–58 years old, male, in good health) were chosen by pre-examination and randomly allotted to the procyanidin and placebo groups. The following criteria were met by all subjects: they showed male pattern baldness on the scalp, had no dermatological disorders other than male pattern baldness on the scalp, had no other diseases, and were not undergoing any medical treatment. The exclusion criterion was the use by the subjects of hair-growing agents within the previous 6 months. The pattern of baldness was classified according to the Ogata scale,[9] specific to Japanese males (Table 1).

Study schedule

The subjects completed the outcome measures to provide baseline data and were given their first supply of test agents. The procyanidin group subjects (25 men) were treated with 0.7% (w/w) procyanidin agent, and the placebo control group (24 men) was treated with vehicle alone. The test agent (about 2 mL per dose) was applied to the subjects' affected scalp area twice daily, giving a daily dose of 18.7 mg of procyanidin oligomers. No use of other hair care products, apart from ordinary shampoos and rinses, was permitted during the clinical trial. The tests were performed in a double-blind fashion. In the first 6 months, we compared the procyanidin group and the placebo group to assess any medicinal effects of procyanidin oligomers. After completion of the 6-month period of twice-daily application of these agents, they returned for their visit to complete the outcome measures. The hair-growing effects were evaluated according to the macrophotographically recorded changes in the number of hairs in the designated scalp area. The application time of the procyanidin group was subsequently extended to 12 months, and the time course of the remedial value was examined. After completion of the 12-month period of twice-daily ap-

plication of the agent, they returned for their visit to complete the testing of outcome. The hair-growing effects were evaluated by the macrophotographically recorded changes in the number of hairs in the designated scalp area.

Determination of change in hair density

Before the test, 6 months later, and 12 months later, hairs at a predetermined site (a round area 1 cm in diameter) were clipped with small straight surgical scissors.[10] The site was selected from the outskirts of the affected area on the vertex of each subject using a plastic template connected by a strut to the frame of a pair of eyeglasses, for the purpose of precisely identifying specific scalp area. The hair-cutting sites were photographed using a camera (OM-4 Ti, Olympus Op-

Table 1 Profile of the subjects.

	Baldness patterns (Ogata scale*)			
Group	Type I	Type II	Type III	Type IV
Placebo	2	11	0	9
Procyanidins	2	8	0	11

*Normal:  ; type I:  ; type II:  ; type III:  ; type IV:  .

tical Co., Japan) fitted with a macro lens (Zuiko Auto-macro 38 mm, F2.8) and extension tube 25. The hairs in the photograph of this specific area (a round area 0.8 cm in diameter; area = 0.50 cm^2) at the predetermined site were counted three times each by three independent investigators.

Statistical analysis

The differences in total hair increase after the 6-month trial between the placebo and treatment groups were analyzed using the two-sample t-test. The increase in the total number of hairs after the 12-month trial period was compared with the baseline and a paired t-test was used for the analysis. All differences were considered significant at a level of $P < 0.05$.

Diagnosis by dermatologist

During the course of the 12-month test period, all subjects underwent clinical diagnosis by one of the authors, a dermatologist, who examined the subjects for any adverse dermatological reactions such as inflammation, erythema, or eczema.

Product characteristics

Purified procyanidin oligomers were obtained from unripe apples (Malus pumila Miller var. domestica Schneider, Fuji variety) according to the method described in a previous report.[8] The total procyanidin content was colorimetrically

measured by a method described by Porter et al.[11] and was calculated to be 83.6% (w/w) using procyanidin B-2 as a standard. The product contained 7.3% (w/w) procyanidin B-1, 26.2% (w/w) procyanidin B-2, and 7.7% (w/w) procyanidin C-1 as the major components; other oligomeric procyanidins were also present about 40% (w/w).

Preparation of hair tonic for the clinical trial

The test agent was prepared by dissolving 0.7% (w/w) of procyanidin oligomers from apples in conventional basal solvent including 70% (w/w) of ethanol, 3% (w/w) of 1,3-butylene glycol, 0.15% (w/w) of Nacetylglutamine isostearyl ester, 0.067% (w/w) of citrate-sodium citrate buffer, 0.05% (w/w) of sodium bisulfite, and purified water (the remainder). Vehicle without procyanidin oligomers was used as the placebo control.

Ethical approval

Individual subjects agreed to an informed consent contract. The contract confirmed their willingness to participate in the test, their freedom to drop out at any time, and their willingness to use the agent under the administration of the doctor. The confidentiality of each participant's information was also safeguarded under this contract.

Results and discussion

Withdrawals

Data were available from 43 of the 49 patients who began the trial. Four participants in the procyanidin group and two in the placebo group dropped out for personal reasons. Ultimately, 21 men in the procyanidin group and 22 men in the placebo group were subjected to analysis. No other person dropped out for any reason.

Adverse effects

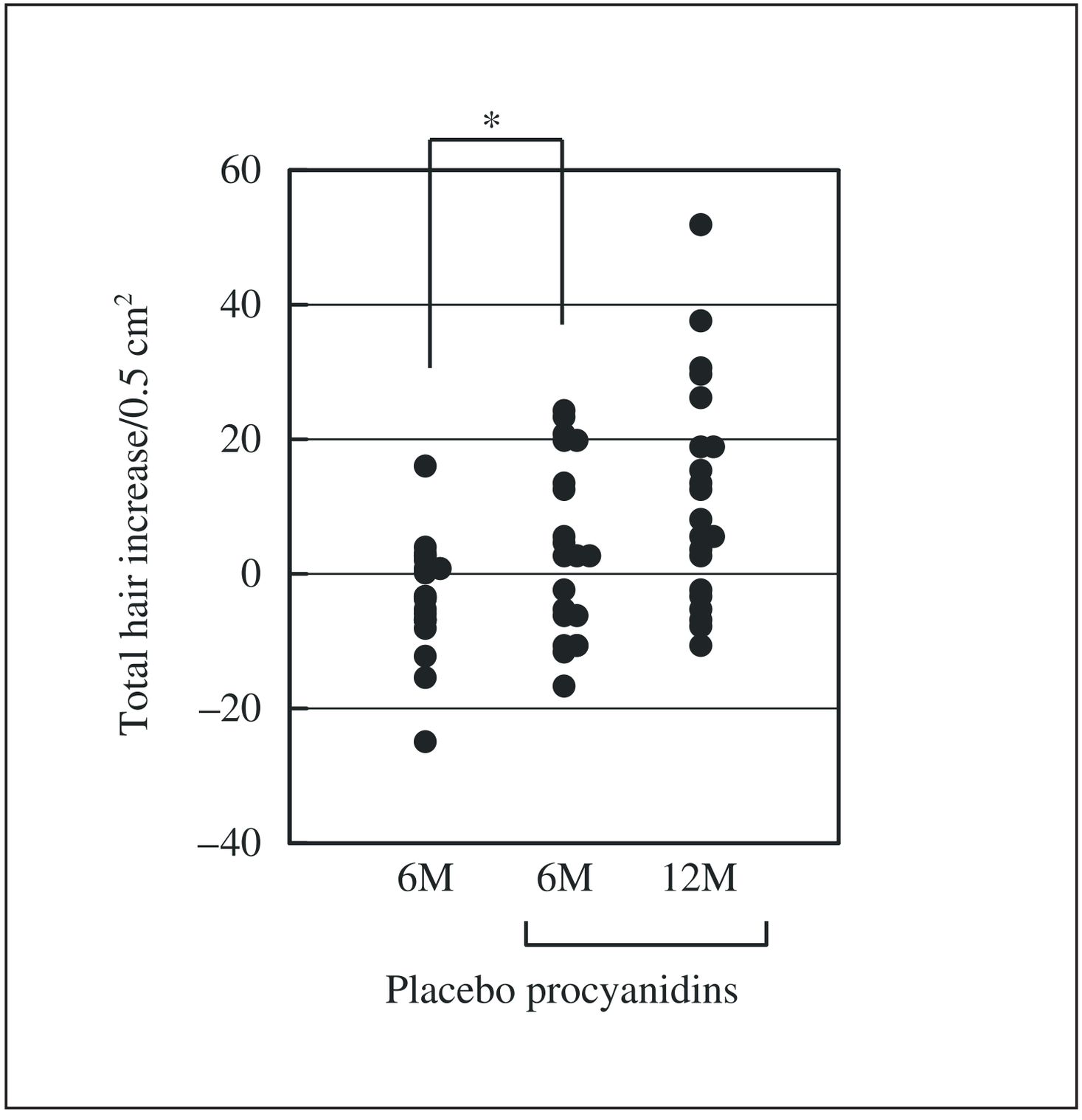
Dermatologic diagnosis revealed that no adverse side effects were caused by the agent, i.e., no inflammation, irritation, or allergic reactions of the scalp were observed in any of the subjects. Furthermore, no subjects complained of itching, pain, dryness, or scaling of the scalp.

Changes in hair density

In the procyanidin group, the increase in number of total hairs in the designated scalp area (a circle 0.8 cm in diameter = 0.50 cm^2) after the 6-month trial was 3.3±13.0 (mean±SD)/0.50 cm^2, whereas in the placebo control group, the increase in number of total hairs was –3.6±8.1 (mean±SD)/0.50 cm^2. It is calculated that the increased number of total hairs in the

designated scalp area of the procyanidin group subjects after the 6-month trial was significantly greater than that of the placebo control group subjects (P<0.001, two-sample t-test) (Fig. 2). The total number of hairs in the designated scalp area after the 12-month procyanidin treatment significantly increased over the baseline value measured at the start of the trial (11.5±16.5 (mean ±SD)/0.50 cm^2; P< 0.005, paired t-test) (Fig. 2). These results show a time-course-dependent improvement in hair density in the procyanidin group subjects.

Figure 2 Change in hair density. The increased number of total hairs in the designated scalp area after the 6-month treatment of placebo control, 6-month treatment of procyanidin agent, and 12-month treatment of procyanidin agent over the baseline value are shown. The increase in total number of hairs in a designated scalp area of the procyanidin group subjects after the 6-month trial was significantly greater than that of the placebo control group subjects (*P<0.001, two-sample t-test). The total number of hairs in the designated scalp area after the 12-month procyanidin treatment significantly increased over the baseline value measured at the start of the trial (P < 0.005, paired t-test).


*
60
40
20
0
–20
–40
Total hair increase/0.5 cm2
6M
6M
12M
Placebo procyanidins

Human clinical trials of topical procyanidins

As the procyanidin oligomers were revealed to possess the highest hair growth-inducing potential of the various proanthocyanidin molecules we had investigated using the murine model,[7] we considered investigating the use of procyanidin oligomers to treat male pattern baldness. We obtained procyanidin oligomers of high purity on an industrial scale from apples for practical use, and conducted a 12-month clinical trial.

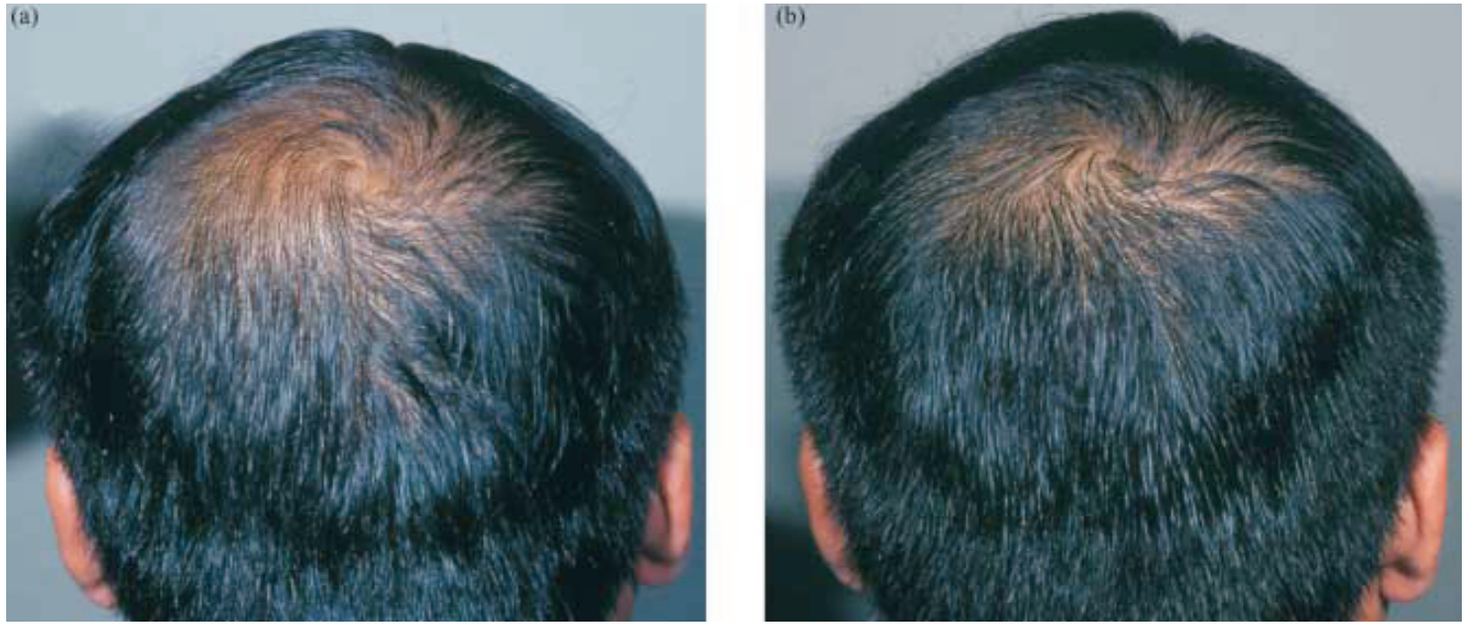


Figure 3 Clinical photographs of a subject before (a) and after (b) 12-month treatment of procyanidin agent.

We observed a total increase of 23 hairs/cm^2 after 12 months of procyanidin treatment. The same effects have been reported with minoxidil[12] and finasteride[13] therapy for male pattern baldness. With the 2% minoxidil treatment, a total increase of 250 hairs/5.1 cm^2 (calculated as a total increase of 49 hairs/cm^2) after 12 months of therapy was reported.[12] With finasteride treatment (1 mg/day, oral administration), a total increase of 86 hairs/5.1 cm^2 (cal-

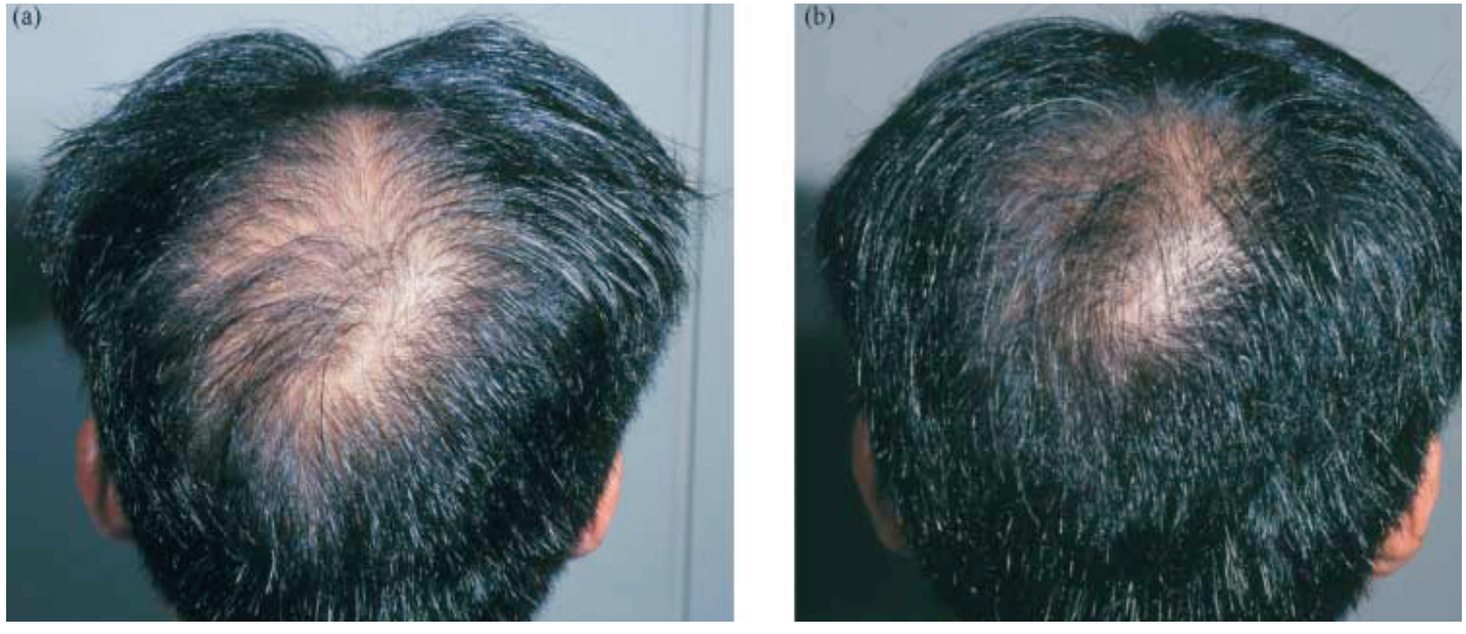


Figure 4 Clinical photographs of a subject before (a) and after (b) 12-month treatment of procyanidin agent.

culated as a total increase of 16.9 hairs/cm^2) after 12 months of therapy was reported.[13] The level of efficacy of our 0.7% procyanidin oligomer preparation was thus considered to compare favorably with both minoxidil and finasteride therapy.

Assumed mechanisms of action

TGF-β has recently been hypothesized to be a catagen-inducing factor,[14,15] and lipid peroxidation[16] and inflammation[17] have been observed to be aggravating factors. As the hair-growing mechanisms of procyanidins, TGF-β-related mechanisms,[18] or mechanisms affected by antioxidation and anti-inflammation are speculated. It appears that the activity of procyanidin oligomers may depend on more than one of their numerous physiological functions.

Effects on hair growth

This investigation provides evidence that procyanidins may impede the development of male pattern baldness. A number of the subjects showed cosmetically satisfactory changes (Figs 3 and 4). This study suggests that the use of procyanidin oligomers to treat male pattern baldness merits further investigation.

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Correspondence: Ayako Kamimura, PhD, Tsukuba Research Laboratories, Kyowa Hakko Kogyo Co., 2, Miyukigaoka, Tsukuba, Ibaraki 305–0841, Japan, E-mail: ayako.kamimura@kyowa.co.jp
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CUTANEOUS BIOLOGY

Procyanidin B-2, extracted from apples, promotes hair growth: a laboratory study

A. KAMIMURA AND T. TAKAHASHI
Tsukuba Research Laboratories, Kyowa Hakko Kogyo Co., 2, Miyukigaoka, Tsukuba, Ibaraki 305–0841, Japan
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Summary

Background We have previously reported that several selective protein kinase C (PKC) inhibitors, including procyanidin B-2, promote hair epithelial cell growth and stimulate anagen induction.
Objectives We discuss the hypothesis that the hair-growing activity of procyanidin B-2 is related to its downregulation

or inhibition of translocation of PKC isozymes in hair epithelial cells.

Methods We examined the effect of procyanidin B-2 on the expression of PKC isozymes in cultured murine hair epithelial cells as well as PKC isozyme localization in murine dorsal skin at different stages in the hair cycle.

Results We observed that procyanidin B-2 reduces the expression of PKC-α, -βI, -βII and -η in cultured murine hair epithelial cells and also inhibits the translocation of these isozymes to the particulate fraction of hair epithelial cells. Our immunohistochemical analyses demonstrated that PKC-α, -βI, -βII and -η are specifically expressed in the outer root sheaths of both anagen and telogen hair follicles. The hair matrix at the anagen stage showed no positive staining for these PKC isozymes. Moderate to intense staining for PKC-βI and -βII in the epidermis and hair follicles was observed in a telogen-specific manner; however, expression of PKC-α and -η during the telogen stage was not conspicuous. Gö 6976, an inhibitor of calcium-dependent (conventional) PKC, proved to promote hair epithelial cell growth.

Conclusions These results suggest that PKC isozymes, especially PKC-βI and -βII, play an important role in hair cycle

progression and that the hair-growing mechanisms of procyanidin B-2 are at least partially related to its downregulation of PKC isozymes or its inhibition of translocation of PKC isozymes to the particulate fraction of hair epithelial cells.

Key words: baldness, hair cycle, hair follicles, procyanidin B-2, protein kinase C

Protein kinase C (PKC) is a major signal transduction pathway in many tissues and cells, and is known to play a key role in cell proliferation, differentiation and regulation.[1] PKC was first identified and characterized by Nishizuka et al. in 1977 as a serine threonine kinase.[2,3] Up to now, at least 12 isozymes have been isolated. PKC is now classified into three major subgroups: (i) contains conventional PKC (α, βI, βII and γ), which is calcium and diacylglycerol dependent; (ii) comprises novel PKC (δ, ε, η and θ) whose activity is calcium independent but diacylglycerol dependent; and (iii) includes atypical PKC (ζ, λ, ι and μ) whose activity is calcium and diacylglycerol independent.[4] PKC is known to act as a differentiation signal in epidermal keratinocytes.[5] However, the role of PKC in hair follicle tissue has not been elucidated. It has been reported that PKC acts

as a negative hair-growing factor[6–9] and that several selective PKC inhibitors, including procyanidins, exhibit hair-growing activity.[9]

There is, however, only limited information on PKC isozyme expression in hair follicles. In human hair follicles, expression of PKC-α, -β, -δ and -ζ has been confirmed in cultured outer root sheath keratinocytes;[10] and in immunohistochemical studies, expression of PKC-α in mice[11] and -η in humans[12,13] has been confirmed in outer root sheaths of hair follicles.

This report describes our investigation of the supposed mechanisms of action of hair-growing activity possessed by procyanidin B-2 from the viewpoint of whether it modulates the expression or translocation of PKC isozymes in hair epithelial cells. We also examined the changes in PKC isozyme expression in murine hair follicles and epidermis in relation to hair cycle progression. In this report, we discuss the hypothesis that the hair-growing activity of procyanidin B-2 is related to its downregulation or inhibition of translocation of PKC isozymes in hair epithelial cells.

Materials and methods

Materials

Procyanidin B-2 [epicatechin-(4b → 8)-epicatechin] (Fig. 1) was obtained from apples according to the method described in a previous report.[14] Polyclonal antibodies against PKC-α, -βI, -βII and -η, -α, -βI and -βII: rabbit antihuman; -η: rabbit antimouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The secondary antibody used was biotinylated goat antirabbit immunoglobulin purchased from DAKO (Glostrup, Denmark). Streptavidin–horseradish peroxidase conjugate was purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, U.K.). Gö 6976 was purchased from Calbiochem-Novabiochem (San Diego, CA, U.S.A.).

Isolation and culturing of murine hair epithelial cells

Murine hair epithelial cells were isolated from 4-dayold C3H / HeNCrj mice (Charles River Japan, Kanagawa, Japan) and cultured in MCDB 153 medium according to the method described in another report.[15]

Immunoblot analysis (Western blotting)

The cultured murine hair epithelial cell pellet was: (i) sonicated in five 10-second bursts in Buffer A [20 mmol L^{-1}

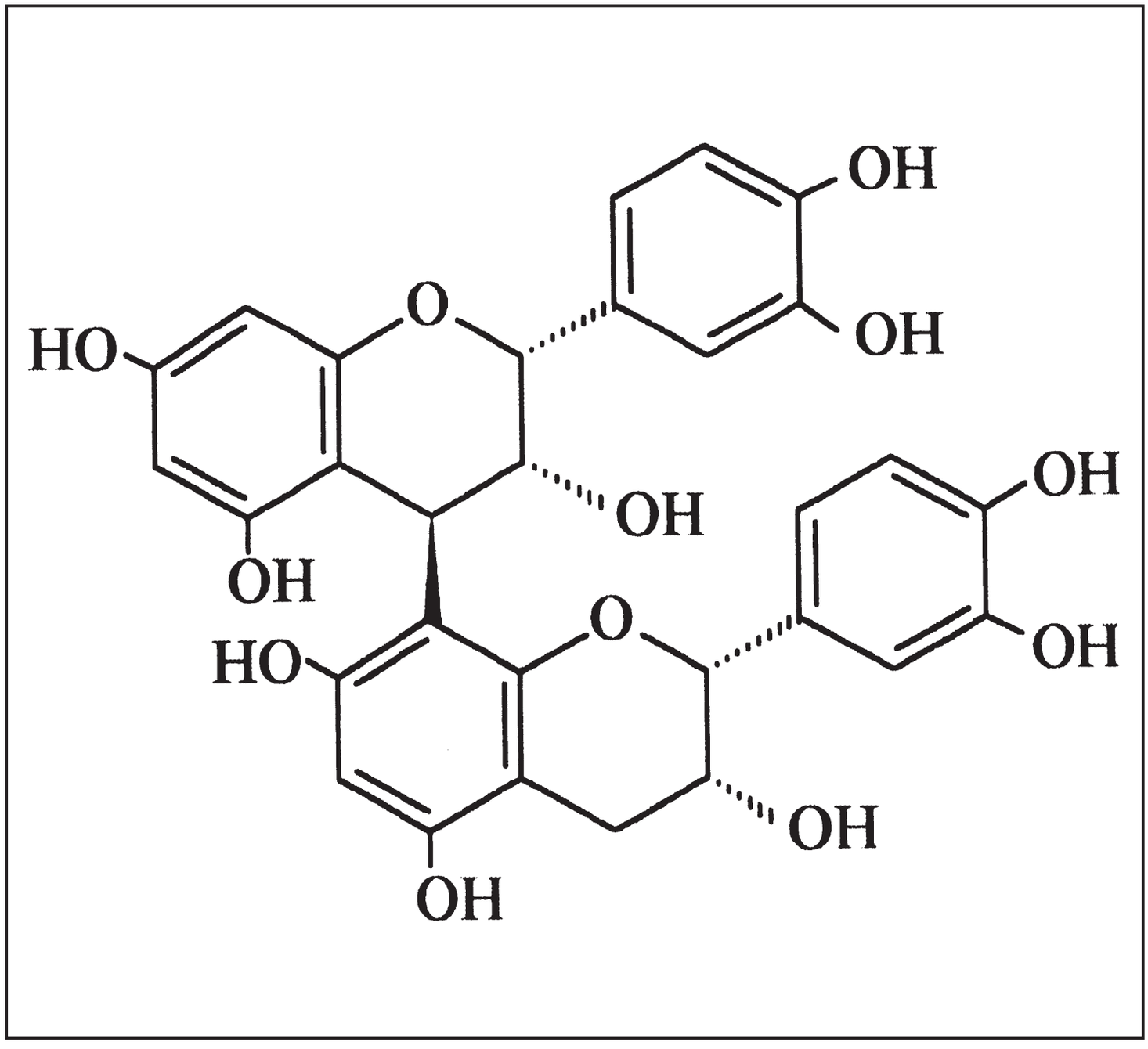


Figure 1. The structure of procyanidin B-2.

Tris(hydroxymethyl)aminomethane (Tris)–HCl (pH 7.5), 2 mmol L^{-1} ethylenediamine tetraacetic acid, 10 mmol L^{-1} ethyleneglycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid, 0.25 mol L^{-1} sucrose, 2 mmol L^{-1} phenylmethylsulphonyl fluoride, 10 μg mL^{-1} leupeptin and 10 mmol L^{-1} 2-mercaptoethanol; final concentrations], and (ii) centrifuged at 100 000 × g for 60 min (4 °C). The supernatants were concentrated to 1 / 10 volume using an ultrafilter (MW 30 000 cutting, UFP2 TTK, Millipore, MA, U.S.A.). The fraction is referred to as a cytosol fraction. The pellets were then: (i) dissolved in Buffer B [Buffer A + 0.5% 1(w/v) polyoxyethylene (10) octylphenyl ether (Triton X-100®]; (ii) sonicated in five 10-s bursts; and (iii) centrifuged at 100 000 × g for 60 min (4°C). The supernantants were concentrated to 1/10 volume using an ultrafilter (MW 30 000 cutting UFP2 TTK, Millipore). The fraction is referred to as a particulate fraction. Protein concentrations were determined spectrophotometrically using a DC-Protein Assay kit (Bio-Rad, Hercules, CA, U.S.A.). Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed according to the method of Laemmli.[16] The proteins in the gel were electroblotted on to a nitrocellulose membrane (Schleicher, Schuell & Keene, NH, U.S.A.) using

a submarine transfer apparatus (Trans-Blot Cell®, Bio-Rad) for 3 h at 60 V per 320 cm^2. The membranes were incubated with diluted polyclonal antibodies (× 500 dilution by the blocking solution) against PKC isozymes (-α, -βI, -βII and -η; Santa Cruz Biotechnology). The membranes were then incubated with biotinylated goat antirabbit immunoglobulin [× 3000 dilution by phosphate-buffered saline (PBS)-T] and with streptavidin–horseradish peroxidase conjugate (× 1000 dilution with PBS-T). Detection of immunoreactive protein was achieved by chemiluminescence using the ECL Western blotting detection system (Amersham Pharmacia Biotech) and recorded by exposure of X-ray film (RX-U, Fuji Photo Film, Tokyo, Japan). Protein bands were identified as PKC by their molecular weight, comigration with their standard proteins (PKC-α, -βI, -βII and -η; human recombinant; Calbiochem-Novabiochem) and lack of staining by the secondary antibody when the primary antibody was omitted. Quantitative analysis of PKC-isozyme expression was performed by densitometry (CS-9000, Shimadzu, Kyoto, Japan).

Colorimetric assay for cell proliferation by MTT

The degree of cell growth was determined by means of an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

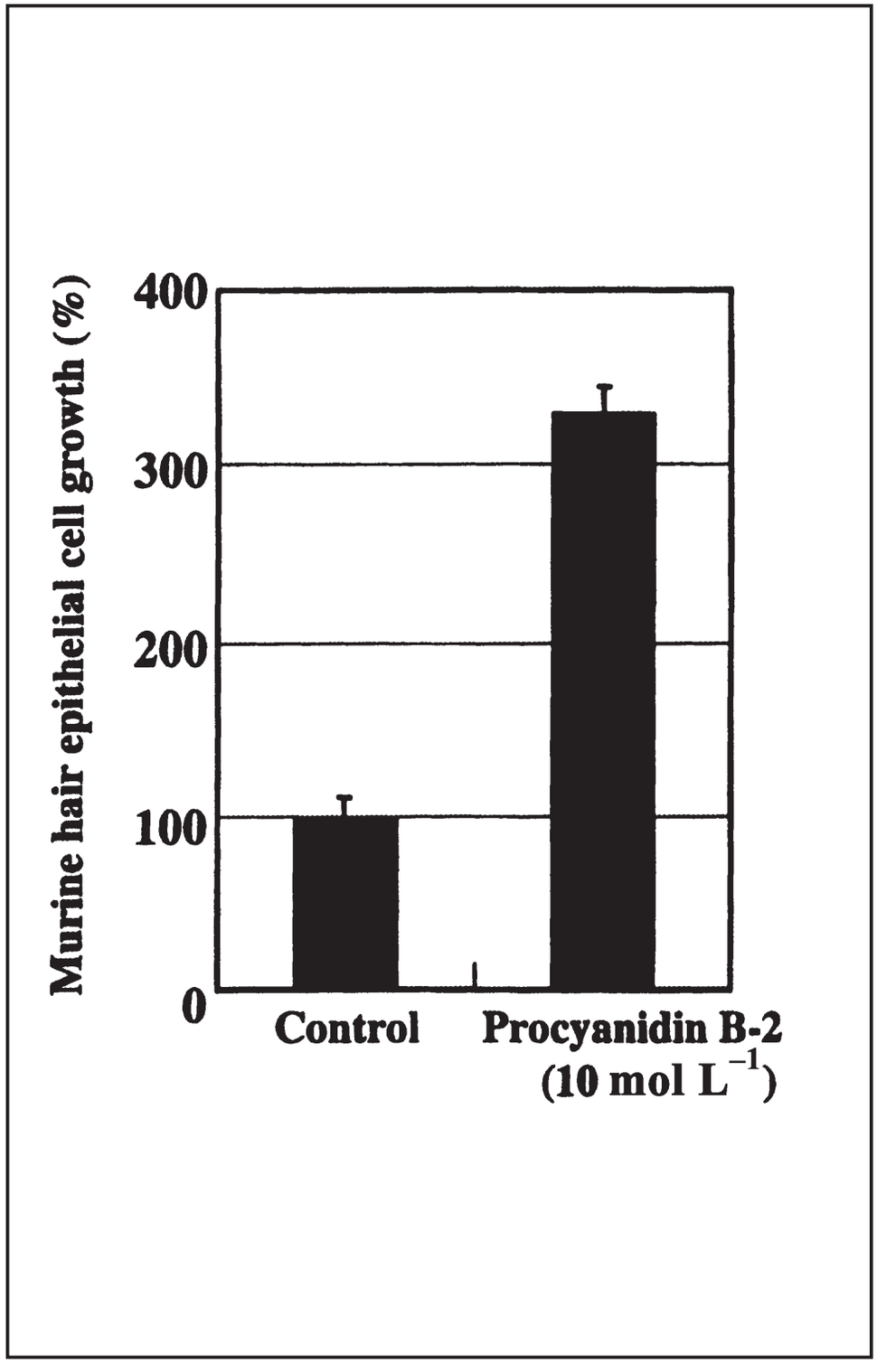


Figure 2.
Procyanidin B-2 markedly promotes growth of hair epithelial cells. Growth-promoting activity for hair epithelial cells relative to controls (= 100%) is shown. Procyanidin B-2 was added to the culture during the last 5 days. For the control, a medium without procyanidin B-2 was used. Results are represented as the mean ± SD (n = 6).

bromide] assay. The details of the procedure are described in another report.[15]

Immunohistochemical staining

Paraffin-embedded tissue sections of C3H / HeSlc mouse (male, Japan SLC, Shizuoka, Japan) back skin were incubated with the primary polyclonal antibody against PKC-α, -βI, -βII and -η [Santa Cruz Biotechnology; diluted 1 : 100 using 5% (w / v) skim milk and 0.1% (w / v) Tween® 20 in PBS] after incubation with 10% (v / v) normal goat serum. Next, they were treated with biotinylated secondary antibody (goat antirabbit), incubated with streptavidin–horseradish peroxidase conjugate, and reacted with 3-amino-9-ethylcarbazole 2(AEC) solution (Histostain-SP™ Kit, Zymed, San Francisco, CA, U.S.A.) and hydrogen peroxide. Next, the specimens were counterstained with haematoxylin. Negative controls were obtained by omission of primary antibody.

Results

Procyanidin B-2 intensively promotes hair epithelial cell growth

We examined the growth-promoting activity on murine hair epithelial cells by procyanidin B-2, and confirmed that procyanidin B-2 shows a high growthpromoting activity of

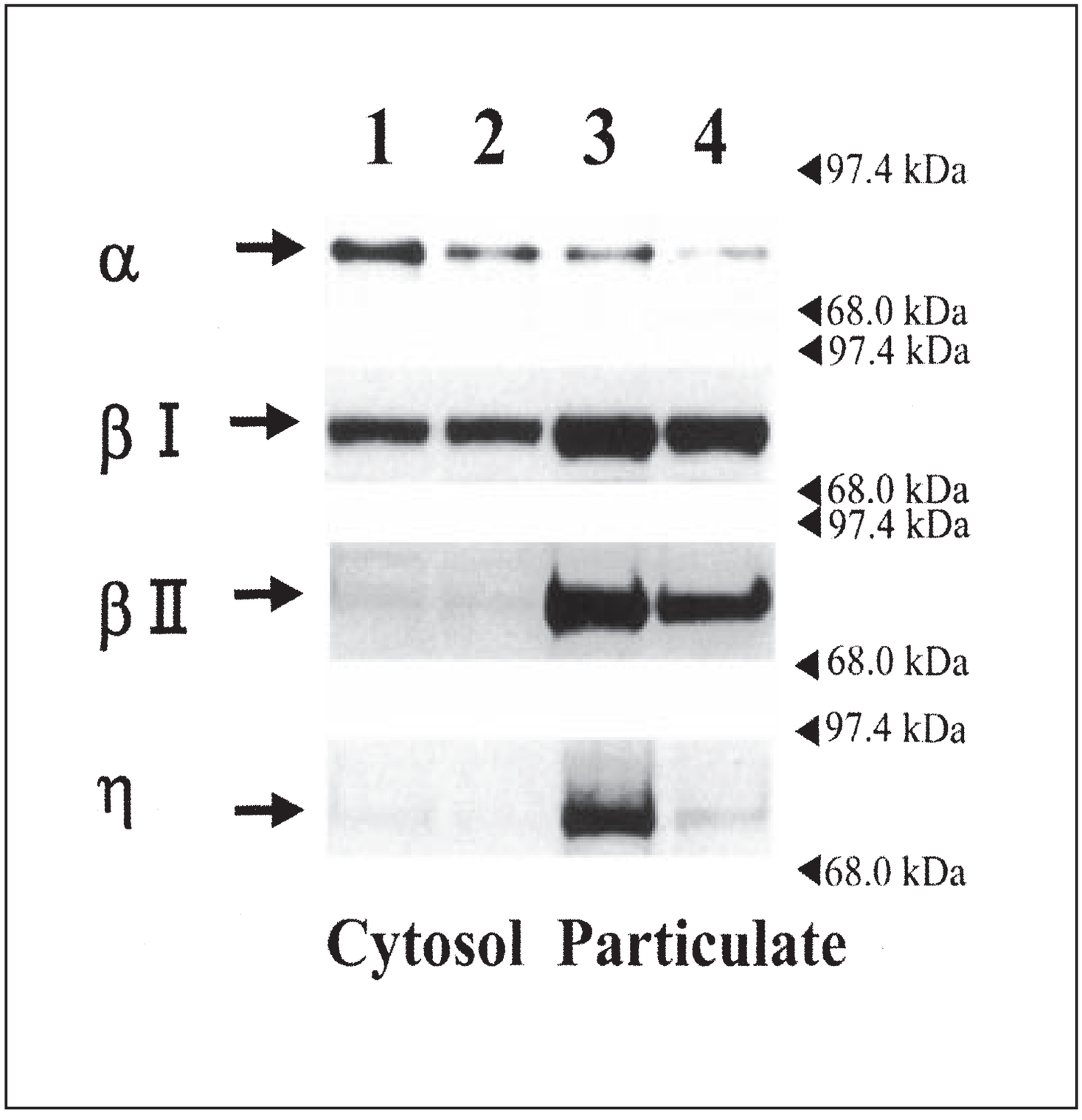
more than 300% (30 μmol L^{-1}) relative to controls (= 100%) in a 5-day culture of hair epithelial cells (Fig. 2).

Procyanidin B-2 decreases the levels of PKC-α, -βI, -βII and -η in both the cytosol and particulate fraction of cultured murine hair epithelial cells

We examined the effect of procyanidin B-2 on the expression of PKC isozymes in cultured murine hair epithelial cells using western blotting. The calcium concentration of the culture medium was raised from 0.03 mmol L^{-1} to 0.5 mmol L^{-1} on day 3 during the 7-day culture period. The hair epithelial cells were incubated in media containing 10 lmol L^{-1} of procyanidin B-2 for the final 96 h of the 7-day culture period. Intense staining for PKC-α and -βI was observed in the cytosol fraction of cultured hair epithelial cells; only weak staining for PKC-βII and -η was observed in the cytosol fraction of cultured hair epithelial cells. Intense staining for PKC-α, -βI, -βII and -η was observed in the particulate fraction of cultured hair epithelial cells. We observed decreases in the levels of PKC-α, -βI, -βII and -η in the cytosol fraction of hair epithelial cells cultured in media containing 10 μmol L^{-1} of procyanidin B-2: (i) α = from 72.9% (procyanidin B-2 = 0 μmol L^{-1}) to 34.2% (procyanidin B-2 = 10 μmol L^{-1}) ($P < 0.02$,

two-sample t-test); (ii) βI = from 34.6% to 30.6%; (iii) βII = from 4.1% to 0.9%; and (iv) η = from 8.6% to 2.6%. (The level of overall expression of each PKC isozyme in the controls, i.e. procyanidin B-2 = 0 μmol L^{-1}, is represented as 100%.) We observed decreases in the levels of PKC-α, -βI, -βII and -η in the particulate fraction of hair epithelial cells cultured in media containing 10 μmol L^{-1} of procyanidin B-2: (i) α = from 27.1% (procyanidin B-2 = 0 μmol L^{-1}) to 12.5% (procyanidin B-2 = 10 μmol L^{-1}); (ii) βI = from 65.4% to 48.3% ($P < 0.05$, two-sample t-test); (iii) βII = from 95.9% to 58.5% ($P < 0.05$, two-sample t-test); and (iv) η = from 91.4% to 12.9% ($P < 0.05$, two-sample t-test). (The level of overall expression of each PKC isozyme in the controls, i.e. procyanidin B-2 = 0 μmol L^{-1}, is represented as 100%.) As a result of the addition of 10 μmol L^{-1} of procyanidin B-2, the overall expression of PKC-α, -βI, -βII and -η in hair epithelial cells decreased: (i) α = from 100% (procyanidin B-2 = 0 μmol L^{-1}) to 46.7% (procyanidin B-2 = 10 μmol L^{-1}) ($P < 0.02$, two-sample t-test); (ii) βI = from 100% to 78.9% ($P < 0.02$, two-sample t-test); (iii) βII = from 100% to 59.4% ($P < 0.002$, two-sample t-test); (iv) η = from 100% to 15.5% ($P < 0.05$, two-sample t-test). (The level of overall expression of each PKC isozyme in the

Figure 3. Procyanidin B-2 decreases the levels of protein kinase C (PKC)-α, -βI, -βII, and -η in both the cytosol and particulate fractions of cultured murine hair epithelial cells. Western blotting analytical results are shown for PKC-α, -βI, -βII and -η in cytosol and particulate fractions extracted from cultured murine hair epithelial cells. Procyanidin B-2 (10 μmol L^{-1}) was added to the culture medium during the final 96 h of the 7-day culture period. Procyanidin B-2 dissolved in purified water was added at a rate of 1% (v / v) to the culture medium. The calcium concentration of the culture medium was raised from 0.03 mmol L^{-1} to 0.5 mmol L^{-1} on day 3 during the 7-day culture period. The data show the cytosol fraction of the control (lane 1), the cytosol fraction of 10 μmol L^{-1} procyanidin B-2 (lane 2), the particulate fraction of the control (lane 3), and the particulate fraction of 10 μmol L^{-1} procyanidin B-2 (lane 4). Specific immunoreactive 80 kDa bands for PKC-α, -βI, -βII and -η were detected. Typical results are shown in three independent experiments performed.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97.4 kDa
α
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β I
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β II
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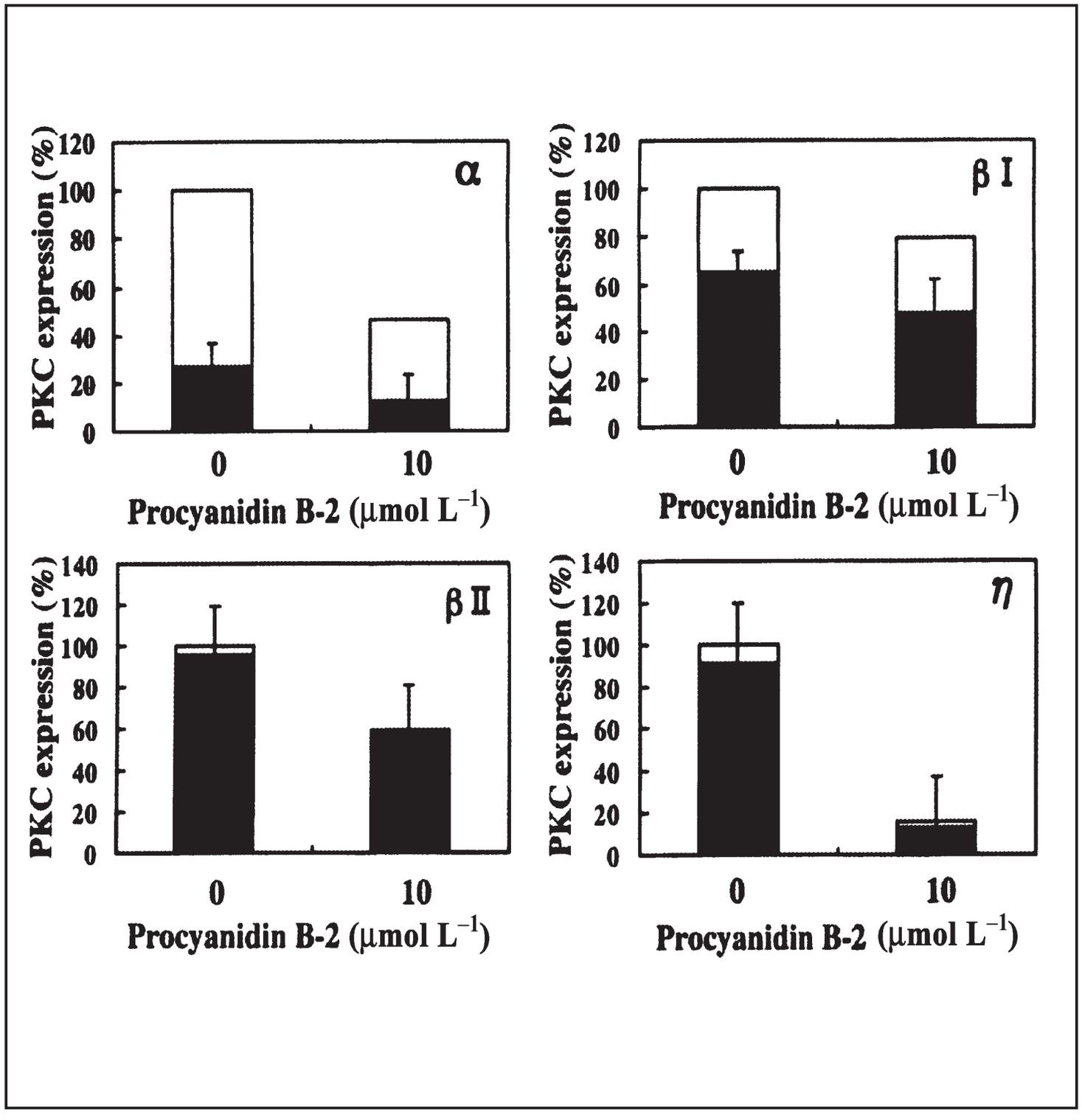
Cytosol Particulate


α
PKC expression (%)
120
100
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Procyanidin B-2 (μmol L−1)
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0
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10
Procyanidin B-2 (μmol L−1)
β II
PKC expression (%)
140
120
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80
60
40
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10
Procyanidin B-2 (μmol L−1)
η
PKC expression (%)
140
120
100
80
60
40
20
0
0
10
Procyanidin B-2 (μmol L−1)

Figure 4. Densitometric analysis of the Western blotting results. Procyanidin B-2 decreases the levels of protein kinase C (PKC)-α, -βI, -βII and -η in both the cytosol and particulate fraction of cultured murine hair epithelial cells; and also suppresses the overall expression of PKC-α, -βI, -βII and -η in cultured murine hair epithelial cells. Procyanidin B-2 (10 μmol L^{-1}) was added to the culture medium during the final 96 h of the 7-day culture period. The calcium concentration of the culture medium was raised from 0.03 mmol L^{-1} to 0.5 mmol L^{-1} on day 3 during the 7-day culture period. (a) PKC-α, (b) PKC-βI, (c) PKC-βII, (d) PKC-η. Clear bar, cytosol fraction; solid bar, particulate fraction; overall = cytosol fraction (clear bar) + particulate fraction (solid bar). The level of overall expression of each PKC isozyme in the controls (procyanidin B-2 = 0 μmol L^{-1}) is represented as 100. Values are represented as mean (for cytosol fractions and overall) or mean ± SD (for particulate fractions) of three independent experiments.

controls, i.e. procyanidin B-2 = 0 μmol L^{-1}, is represented as 100%.) (Figs 3 and 4).

Immunohistochemical study of protein kinase C-α, -βI, -βII and -η in murine hair follicles at different stages in the hair cycle

In 3-day-old dorsal skin in the anagen stage, only weak staining for PKC-α was observed in the outer root sheath keratinocytes below the bulge area (data not shown). In 3.5-week-old and 4.5-week-old dorsal skin, weak staining for PKC-α was observed in the basal and spinous layer of the epidermis. In the hair follicles of 3.5-week-old dorsal skin in the telogen stage, moderate staining for PKC-α was observed in the infundibulum of the outer root sheath keratinocytes (Fig. 5a). In the hair follicles of 4.5-week-old dorsal skin in the anagen stage, intense staining for PKC-α was observed in the bulge area of the outer root sheath keratinocytes, but no staining for PKC-α was observed in the hair matrix (Fig. 5b).

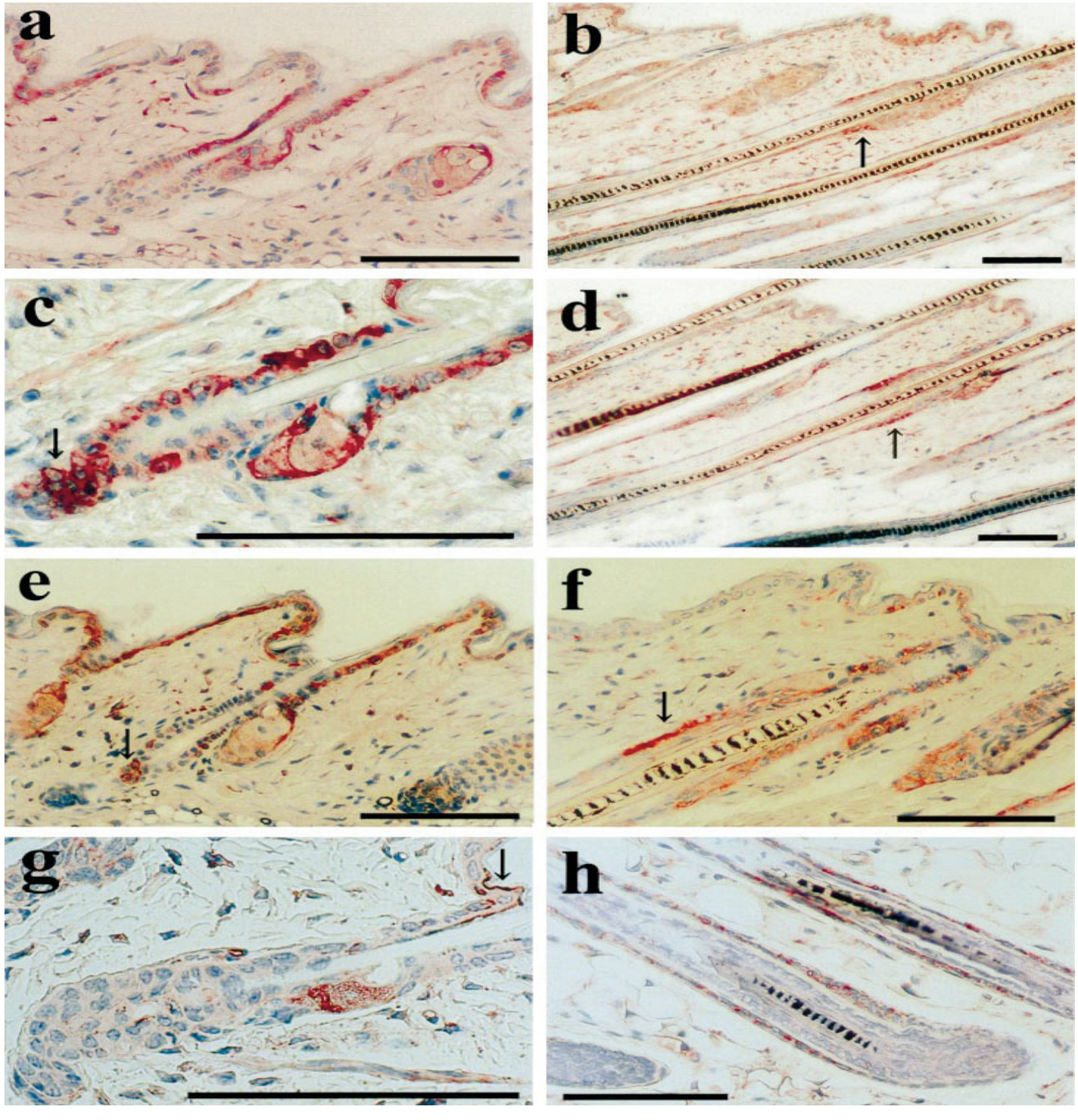
In 3-day-old dorsal skin in the anagen stage, only weak staining for PKC-βI was observed in the bulge area of the outer root sheath keratinocytes (data not shown). In 3.5-week-old dorsal skin in the telogen stage: (i) intense staining for PKC-βI was observed in the infundibulum of the outer root sheath keratinocytes and the hair germ, and (ii) moder-

ate staining for PKC-βI was observed in the basal layer of the epidermis and the outer root sheath keratinocytes below the sebaceous gland in the hair follicles (Fig. 5c). In 4.5-week-old dorsal skin in the anagen stage: (i) scattered staining for PKC-βI was observed in the basal layer of the epidermis and the infundibulum of the outer root sheath keratinocytes; (ii) intense staining for PKC-βI was observed in the bulge area of the outer root sheath keratinocytes; (iii) weak staining for PKC-βI was observed in the outer root sheath keratinocytes below the bulge area; and (iv) no staining for PKC-βI was observed in the hair matrix (Fig. 5d).

In 3-day-old dorsal skin in the anagen stage, only weak staining for PKC-βII was observed in the bulge area of the outer root sheath keratinocytes (data not shown). In 3.5-week-old dorsal skin in the telogen stage: (i) intense staining for PKC-βII was observed in the basal layer of the epidermis, in the infundibulum of the outer root sheath keratinocytes and in the hair germ, and (ii) weak staining for PKC-βII was observed in the outer root sheath keratinocytes below the sebaceous gland (Fig. 5e). In 4.5-week-old dorsal skin in the anagen stage: (i) scattered staining for PKC-βII was observed in the basal layer of the epidermis and the infundibulum of

the outer root sheath keratinocytes; (ii) intense staining for PKC-βII was observed in the bulge area of the outer root sheath keratinocytes; (iii) weak staining for PKC-βII was observed in the outer root sheath keratinocytes below the bulge

Figure 5. Immunohistochemical staining for PKC-α, -βI, -βII and -η in murine dorsal skin at different stages in the hair cycle: (a) 3.5-week-old C3H mouse dorsal skin (telogen stage) stained for PKC-α; (b) 4.5-week-old C3H mouse dorsal skin (anagen stage) stained for PKC-α (arrow indicates the bulge area of hair follicle); (c) 3.5-week-old C3H mouse dorsal skin (telogen stage) stained for PKC-βI (arrow indicates the hair germ of hair follicle); (d) 4.5-week-old C3H mouse dorsal skin (anagen stage) stained for PKC-βI (arrow indicates the bulge area of hair follicle); (e) 3.5-week-old C3H mouse dorsal skin (telogen stage) stained for PKC-βII (arrow indicates the hair germ of hair follicle); (f) 4.5-week-old C3H mouse dorsal skin (anagen stage) stained for PKC-βII (arrow indicates the bulge area of hair follicle); (g) 3.5-week-old C3H mouse dorsal skin (telogen stage) stained for PKC-η (arrow indicates the granular layer of epidermis); (h) 4.5-week-old C3H mouse dorsal skin (anagen stage) stained for PKC-η. Bar = 100 μm.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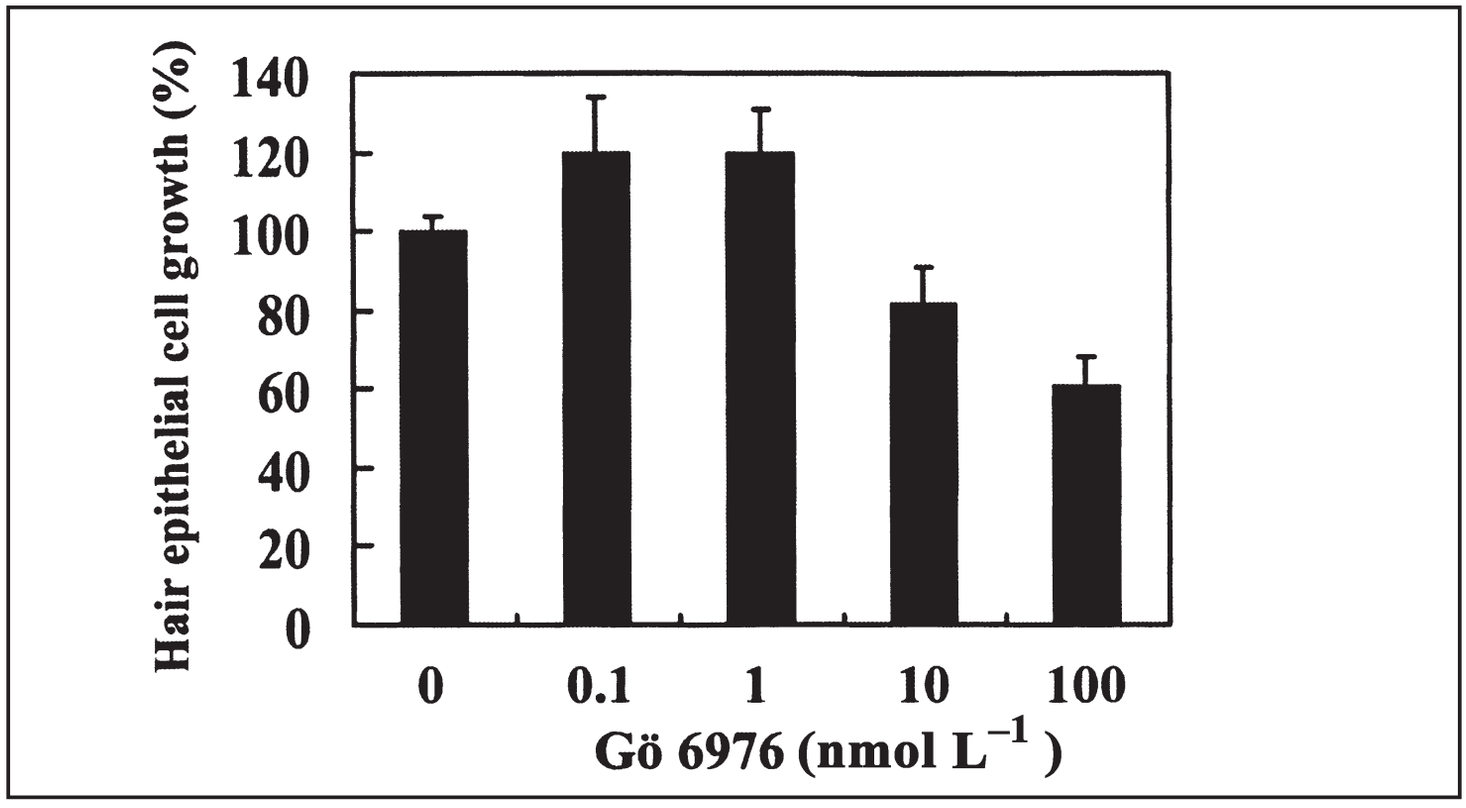

Figure 6. Gö 6976 promotes the growth of cultured murine hair epithelial cells. Growth-promoting activities relative to controls (= 100%) are shown. Gö 6976 dissolved in dimethyl sulphoxide was added at a rate of 1% (v / v) to the culture medium during the 5-day culture period. For the control, we used a medium to which dimethyl sulfoxide was added at the same rate of 1% (v / v). Results are represented as mean ± SD (n = 6) carried out with primary cultures prepared from 50 neonatal mice. These results were confirmed in an additional experiment.

area; and (iv) no staining for PKC-βII was observed in the hair matrix (Fig. 5f).

In these three stages examined (3-day-old, 3.5-week-old and 4.5-week-old), intense staining for PKC-η was observed in the granular layer of the epidermis. In the hair follicles of 3-day-old dorsal skin in the anagen stage, scattered staining for PKC-η was observed in the outer root sheath keratinocytes (data not shown). In the hair follicles of 3.5-week-old dorsal skin in the telogen stage, weak staining for PKC-η was observed in the whole outer root sheath keratinocytes (Fig. 5g). In the hair follicles of 4.5-week-old dorsal skin in the anagen stage, intense staining for PKC-η was observed in the bulge area of the outer root sheath keratinocytes, but no staining for PKC-η was observed in the hair matrix (Fig. 5h).

Gö 6976 promotes the growth of hair epithelial cells at the optimum concentration

It is reported that Gö 6976 selectively inhibits PKC-α,[17] -βI[17] and -μ.[18] We examined the effects of Gö 6976 on hair epithelial cell growth. Our results confirmed that Gö 6976 promotes cultured murine hair epithelial cell growth at about 120% relative to controls over the Gö 6976 concentration range of 0.1–1.0 nmol L^{-1}. Hair epithelial cell growth

was inhibited above a Gö 6976 concentration of 10 nmol L^{-1} (Fig. 6).

Discussion

Procyanidin B-2 inhibits protein kinase C

Procyanidin B-2 is a polyphenol compound classified as a proanthocyanidin, whose structure comprises epicatechin dimmers linked in the 4β → 8 connection mode. It is commonly found in plant species such as apples and grape seeds. We have previously reported and also confirmed in this report that procyanidin B-2 intensively promotes murine hair epithelial cell growth at a rate of about 300% relative to controls (Fig. 2) and stimulates anagen induction in vivo;[14] and its hairgrowing mechanisms are speculated to be linked to its selective PKC inhibition.[9] Procyanidin B-2 has been shown to inhibit PKC in enzyme assay systems using rat brain PKC, showing an IC_{50} (= 50% inhibiting constant) value of 8.6 μmol L^{-1}.[19]

Protein kinase C regulates cell differentiation and proliferation

PKC has been suggested as a potential mediator for signal transduction in cell regulation in relation to differentiation, proliferation and apoptosis.[1,20] PKC is known to act as a

differentiation signal in epidermal keratinocytes. It has been reported that in epidermal keratinocytes, PKC activation is essential in differentiation in the course of keratinization.[5] It has been reported that PKC-α,[21–24] -β25 and -η[12,26] are assumed to play a role in epidermal keratinocyte differentiation. It is known that PKC exerts a negative influence on the growth of some types of cells such as vascular smooth muscle cells (rat[27]), a breast adenocarcinoma cell line (MCF-7, human[28]), a colon cancer cell line (HT-29, human[29]), bone marrow-derived mast cells (mouse[30]) and a mammary epithelial cell line (HC11, mouse, PKC-α and -βI[31]). However, there is as yet limited information on the role of PKC in hair follicles.

Procyanidin B-2 decreases the level of protein kinase-α, -βI, -βII and -η in murine hair epithelial cells

We examined for the first time the effects of procyanidin B-2, a known specific PKC inhibitor, on PKC isozyme expression and translocation in hair epithelial cells. Our results indicate that procyanidin B-2 decreases the levels of PKC-α, -βI, -βII and -η in both the cytosol and particulate fraction of cultured murine hair epithelial cells. It is known that the distribution of PKC undergoes changes during cell activation.

Table 1. The localization of PKC-α in murine dorsal skin at different stages in the hair cycle

Age	3 days	3.5 weeks	4.5 weeks
Hair cycle stage	Anagen	Telogen	Anagen
Epidermis			
Granular layer	None	None	None
Spinous layer	None	Weak	Weak
Basal layer	None	Weak	Weak
Hair follicle			
Outer root sheath			
Infundibulum[a]	None	Moderate	None
Below the sebaceous gland[b]	–	Weak	–
Bulge area[c]	None	–	Intense
Below the bulge area[c]	Weak	–	Moderate
Hair germ[b]	–	None	–
Hair matrix[c]	None	–	None
Dermal papilla	None	None	None

[a]For anagen and telogen, [b]for telogen, [c]for anagen.

Table 2. The localization of PKC-βI in murine dorsal skin at different stages in the hair cycle

Age	3 days	3.5 weeks	4.5 weeks
Hair cycle stage	Anagen	Telogen	Anagen
Epidermis			
Granular layer	None	None	None
Spinous layer	None	None	None
Basal layer	None	Moderate	Scatterable
Hair follicle			
Outer root sheath			
Infundibulum[a]	None	Intense	Scatterable
Below the sebaceous gland[b]	–	Moderate	–
Bulge area[c]	Weak	–	Intense
Below the bulge area[c]	None	–	Weak
Hair germ[b]	–	Intense	–
Hair matrix[c]	None	–	None
Dermal papilla	None	None	None

[a]For anagen and telogen, [b]for telogen, [c]for anagen.

Table 3. The localization of PKC-βII in murine dorsal skin at different stages in the hair cycle

Age	3 days	3.5 weeks	4.5 weeks
Hair cycle stage	Anagen	Telogen	Anagen
Epidermis			
Granular layer	None	None	None
Spinous layer	None	None	None
Basal layer	None	Intense	Scatterable
Hair follicle			
Outer root sheath			
Infundibulum[a]	None	Intense	Scatterable
Below the sebaceous gland[b]	–	Weak	–
Bulge area[c]	Weak	–	Intense
Below the bulge area[c]	None	–	Weak
Hair germ[b]	–	Intense	–
Hair matrix[c]	None	–	None
Dermal papilla	None	None	None

[a]For anagen and telogen, [b]for telogen, [c]for anagen.

Table 4. The localization of PKC-η in murine dorsal skin at different stages in the hair cycle

Age	3 days	3.5 weeks	4.5 weeks
Hair cycle stage	Anagen	Telogen	Anagen
Epidermis			
Granular layer	Intense	Intense	Intense
Spinous layer	None	Weak	None
Basal layer	None	Weak	None
Hair follicle			
Outer root sheath			
Infundibulum[a]	Scatterable	Weak	Weak
Below the sebaceous gland[b]	–	Weak	–
Bulge area[c]	Scatterable	–	Intense
Below the bulge area[c]	Scatterable	–	Moderate
Hair germ[b]	–	None	–
Hair matrix[c]	None	–	None
Dermal papilla	None	None	None

[a]For anagen and telogen, [b]for telogen, [c]for anagen.

It is thought that procyanidin B-2 affects the intracellular localization of PKC-α, -βI, -βII and -η and modulates interactions with membranes, the cytoskeleton, and with distinct subcellular compartments, followed by initiation of cellular reactions such as mitogenesis.

The localization of protein kinase C isozymes in skin

As for the localization of PKC isozymes in skin, expression of PKC-α, -β, -γ, -δ, -ε, -η, -ζ and -μ in murine epidermis has been reported: (i) α: C57BL / 6 mice;[11] (ii) α, β and γ: C57BL / 6 mice and Sencar mice;[32] (iii) η: CD-1 mice;[33] (iv) η and ζ: NMRI mice;[34] (v) α, β, δ and ε: CD-1 mice;[35] (vi) α, βII, δ, ε and ζ: CD-1 mice;[36] (vii) α, β, γ, δ, ε and ζ: Sencar mice;[37,38] and (ix) α, β, δ, ε, η and ζ: CD-1 mice;[39] (x) α, δ, ε, η, ζ and μ: NMRI mice.[40] In murine cultured epidermal keratinocytes, expression of PKC-α, -β, -δ, -ε, -η and -ζ has been reported: (i) α, δ, ε, η and ζ: BALB / c mice;[41,42] (ii) α, δ, η and ζ: BALB/ c mice;[43] and (iii) α, β, δ and ζ: CD-1 mice.[44] Abundant localization of PKC-β in the Langerhans cells in mice has also been reported (C57BL / 6 mice;[45] CD-1 mice[46]). Wang and Smart[11] observed the expression of PKC-α in the outer root sheaths of murine hair follicles. Little is known about the localization of PKC isozymes in murine hair follicles.

In our experiments, positive staining was observed in the basal (PKC-α), spinous (PKC-α) (Fig. 5b) and granular (PKC-η) (Fig. 5h) layers of the epidermis in the anagen stage, consistent with other reported results (α;[11] η[33]). We observed the expression of PKC-α in the hair follicles (Fig. 5a,b) consistent with other reported results.[11] For PKC-βII, scattered staining was observed in the basal layers of the epidermis in the anagen stage (4.5 weeks old) (Fig. 5f) consistent with reported results for Langerhans cells (β;[45] βII[46]). PKC-β is also known to be involved in murine melanogenesis.[47] However, too little is known to enable PKC-β to be discussed separately as βI or βII with respect to its presence in skin. Concerning the existence of PKC-β in primary cultured murine epidermal keratinocytes, positive[44] and negative[41,43] reports exist. We also confirmed the expression of PKC-β in primary cultured murine hair epithelial cells from the support data in an experiment using a reverse transcriptase–polymerase chain reaction (RT–PCR). We obtained at high frequency a RT–PCR product identical to PKC-β[48] from the cDNA of primary cultured murine hair epithelial cells in an experiment using a set of primers with sequence CGGGGTACCGTXATGGAG and CCGGAATTCCCACCAGTC (data not shown). Conse-

quently, we have confirmed the expression of PKC-α, -βI, -βII and -η in murine hair epithelial cells.

Speculations for the role of protein kinase C in hair cycle progression

We observed the expression of PKC-α, -βI, -βII and -η in the outer root sheaths of both anagen and telogen hair follicles (Tables 1–4): PKC-α, -βI, -βII and -η were specifically expressed with the highest intensity in the bulge area of the outer root keratinocytes of the 4.5-week-old anagen hair follicles; no expression was observed of PKC-α, -βI, -βII or -η in the hair matrix cells in the anagen stage. From the fact that the hair matrix is assumed to be in a highly proliferative state in the anagen stage, it is speculated that PKC-α, -βI, -βII and -η at least are not involved in promoting hair epithelial cell growth. The basal layer of the epidermis and the hair follicles, especially the infundibulum of the outer root sheath keratinocytes and the hair germ, were moderately or intensely stained for PKC-βI and -βII in a telogen-specific manner. We present for the first time the hypothesis that dynamic changes, such as increased expression of PKC-βI and -βII in the epidermis and hair follicles, act to induce and maintain the telogen stage of the hair cycle. This hypothesis is supported by the result that Gö 6976, which is an inhibitor

of calcium-dependent PKC inhibitor, promotes murine hair epithelial cell growth (Fig. 6); this is also supported by the results of an experiment using a PKC-βI-overexpressing murine epidermal keratinocyte cell line (3PC cells) whose results suggest that PKC-βI has a growth inhibitory effect on epidermal keratinocytes.[49]

Results of experiments using several PKC inhibitors or activators suggest that PKC acts as a negative hairgrowing factor.[6–9] Li et al. examined the levels of PKC-α and -δ in BALB/c mice back skin in the course of hair growth induced by diphencyprone[50] and hair plucking;[51] and concluded that the downregulation of PKC-α expression in skin appears to cause anagen induction in the hair cycle progression. Cyclosporin A is an immunosuppressive agent known to cause hirsutism. It is reported that cyclosporin A downregulates the expression of PKC-α and -β[52] and inhibits the activation and translocation of PKC-β to the plasma membrane[53] in human lymphocytes. We examined the effect of cyclosporin A on PKC expression and translocation in murine hair epithelial cells and observed that cyclosporin A reduced the levels of PKC-α, -βI, -βII and -η in the particulate fraction of cultured murine hair epithelial cells.[54] In addition, calphos-

tin C, a selective PKC inhibitor, known to possess hair-growing activity, has been reported to inhibit translocation of PKC-βII in lymphocytes to cytoplasmic aggregates of spectrin, a major cytoskeleton component.[55]

As the hair-growing mechanism of procyanidin B-2, the involvement of its inhibitory effects on the expression of one or more of these PKC isozymes (α, βI, βII and η) in keratinocytes in both skin and hair was considered likely.

Gö 6976, a specific inhibitor of protein kinase C-α and -βI, promotes hair epithelial cell growth

Gö 6976 is reported to inhibit PKC-μ (IC_{50} = 20 nmol L^{-1}) in addition to PKC-α (IC_{50} = 2.3 nmol L^{-1}) and -βI (IC_{50} = 6.2 nmol L^{-1}). Growth-inhibiting effects on hair epithelial cells were observed above a Gö 6976 concentration of 10 nmol L^{-1}, while Gö 6976 promotes hair epithelial cell growth at Gö 6976 concentrations of between 0.1 nmol L^{-1} and 1 nmol L^{-1} (Fig. 6). The growth-inhibiting action caused by Gö 6976 at a higher dose range of above 10 nmol L^{-1} may well derive from its inhibiting action on PKC-μ, as PKC-μ is a PKC isozyme that is known to play a role in promoting epidermal keratinocyte growth.[56] Further investigations into other selective PKC inhibitors, which possess a diverse spectrum of PKC

isozyme inhibiting activities, will provide useful information about which PKC isozymes are most involved in the regulation of progression of the hair cycle.

Conclusions

The results of the experiments reported in this paper suggest that procyanidin B-2, a compound that possesses hair-growing activity, causes modulation of the expression and translocation of PKC isozymes (α, βI, βII and η) in hair epithelial cells. Our results, combined with those of other investigations, suggest a possible link between the hair-growing activity possessed by procyanidin B-2 and its downregulation or inhibition of translocation of PKC isozymes in hair epithelial cells in addition to its PKC inhibiting activity. It is highly probable that PKC plays a key role in hair cycle regulation.

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Correspondence: E-mail: ayako.kamimura@kyowa.co.jp

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CLINICAL DERMATOLOGY • REVIEW ARTICLE

Male androgenetic alopecia

R. Hoffmann
Department of Dermatology, Philipp University, Marburg, Germany

Summary

Androgenetic alopecia (AGA) is the most common type of hair loss in men. The relative strong concordance of the degree of baldness in fathers and sons is not consistent with a smiple Mendelian trait and a polygenic basis is considered to be most likely. So far the predisposing genes for AGA are unknown and we do not understand the molecular steps involved in androgen-dependent beard growth versus androgen-dependent hair loss, but AGA can be defined as a DHT-dependent process with continuous miniaturization

of sensitive hair follicles. The type 2 5aR plays a central role by the intrafollicular conversion of T to DHT. Due to the inceasing knowledge in this field, this article shall privide an critical overwiew of recent discoveries.

Introduction

Androgenetic alopecia (AGA) is the most common type of hair loss in men. This continuous process results in a type of alopecia that follows a definite pattern in those individuals who are genetically predisposed. This androgen-dependent condition is often referred to as male pattern, or common, baldness. The prevalence of progressive AGA approaches 50% of Caucasian men beyond the age of 40 years, whereas in Asian, native American and African–American men the prevalence is lower and AGA is less severe. All the hairs in an affected area may be involved in the miniaturization process and over the time the region may be covered with fine, hardly visible vellus hairs. Along with hair miniaturization the production of pigment ceases. There is still a controversy as to whether the total number of hair follicles decreases during AGA. However, it can be assumed that some hairs in AGA are definitely lost, but the majority

of hair shafts are still present as tiny vellus hairs. The pathogenesis of AGA is not completely understood, but rather recent experimental and clinical advances enable us to explain some steps leading to androgenetic hair loss. Therefore, this review provides a critical account of the current understanding of the etiopathology of AGA in men.

Inheritance

The relative strong concordance of the degree of baldness in fathers and sons is not consistent with a simple Mendelian trait and a polygenic basis is considered to be most likely.[1,2] The predisposing genes are still unknown. The genes for type 1 and type 2 5α-reductase (5α-R) and steroid sulphatase[3,4] are not associated with the inheritance of AGA.[1,5] It has been postulated that polycystic ovaries (PCO) in females and early onset AGA in brothers of those PCO-affected women are associated with one allele of the steroid metabolism gene CYP17 which affects androgen production or action.[6,7] Another susceptibility gene for PCO has been linked to a polymorphism of the insulin gene.[8] In man, the number of CAG repeats is polymorphic and expansion of CAG repeats in the androgen receptor (AR) has clinical implications for human disease.

Shorter CAG-repeat lengths may be associated with the development of androgen-mediated skin disorders in men and women.[9] However, the androgen receptor gene is located on the X chromosome and does not explain father-to-son inheritance. On the other hand, we recently described individuals suffering from adrenoleukodystrophy, an X-chromosomal recessive trait; although affected men tend to have low serum testosterone concentrations, one of the clinical hallmarks is a severe AGA-like hair loss with early onset. We therefore hypothesized that one of the AGA-predisposing genes might be the X-linked gene for adrenoleukodystrophy.[10]

Animal models

Several animal species have been reported to develop hair loss resembling human androgenetic alopecia, including bears, lions and non-human primates. Androgen-dependent hair growth has also been described in the red deer. However, a well-studied nonhuman baldness model is the stump-tailed macaque which is a protected species. These macaques have been used to assess the efficacy of several compounds to treat androgenetic hair loss such as minoxidil, systemic 5α-reductase inhibitors, and topical androgen receptor block-

ers.[11–14] In the larger species, the animals are outbred, and the high costs of maintenance makes them of little practical use as research models. Rodent models can be used in several ways for testing modes of therapy or disease prevention. In male Spraque–Dawley rats it has been shown that the hair growth of the dorsal coat appears to be androgen-dependent. Castration of male rats resulted in an accelerated entry into

Table 1 Different effects of androgens on hair growth.

Androgen-insensitive hair follicles:	These hair follicles grow without the influence of androgens (occipital scalp hair follicles and of the eyelids).
Androgen-dependent hair follicles:	These hair follicles enlarge in response to androgens to grow longer and thicker hairs (e.g. the beard).
Androgen-sensitive hair follicles:	These hair follicles display a shortening of the anagen phase and miniaturization of the hair follicle, which results in the formation of progressively thinner and shorter hair (frontal scalp hair in AGA).

anagen III, whereas supplementation with testosterone inhibited this process.[15] The hair shafts of castrated rats appeared to be thicker and hair loss was not observed. At present only one strain of mice, the androchronogenetic mouse, has been described that displays an AGA-like hair loss. This hair loss can be aggravated by infusion of testosterone or DHT and is on the other hand treatable with minoxidil or cyproterone acetate.[17,18] So far, however, no further studies have been performed by use of this model. Another approach has been to transplant human hair follicles from androgen-dependent sites of the scalp (frontal hair) onto testosteroneconditioned nude mice and to measure the hair cycles of these hair follicles and to assess the effect of several drugs on growth characteristics of these hairs.[16]

Pathogenesis

More than 50 years ago Hamilton observed that men who were castrated did not develop AGA.[19] Therefore, it was concluded that the growth of hair follicles is in some areas androgen-dependent (Table 1). At present it is not known how androgens exert their paradoxic effect on the growth of hair follicles at different body sites, and which genes are involved.

However, Hamilton showed that AGA can be triggerd in castrated men by injecting testosterone.

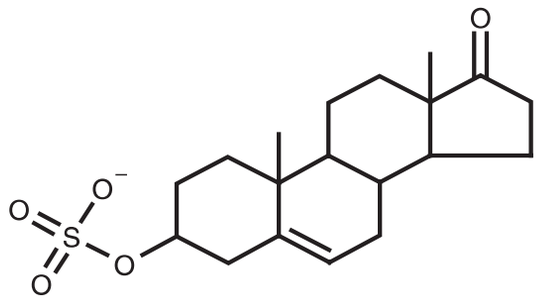
Minimal to no beard growth or AGA is seen in pseudohermaphrodites who lack 5α-reductase, indicating that DHT, the 5α-reduced metabolite of testosterone is the principal mediator of androgen-dependent hair loss. Interestingly, 5α-reduced metabolites of testosterone are increased in balding areas of the human scalp as well as in the scalp of the stump-tailed macaques. It is not yet clear whether DHT is derived from the local metabolism or from the circulation, but it can be assumed that under the influence of DHT hair loss is characterized by a shortening of the anagen phase and miniaturization of the hair follicle, which results in thinner and shorter hair. There is considerable support for the idea that hair follicle size is determined by the size of its dermal papilla. Van Scott[20] demonstrated a constant geometric correlation between the proportions of the human hair follicle, the dermal papilla and the hair bulb matrix. They concluded that the size of the dermal papilla ultimately dictates the size of the growing hair. Hence, in AGA some dermal papilla cells will get lost. The most likely mechanism is by apoptosis, but cell displacement might be an additional explanation.

Androgen metabolism and hair growth

As stated above, androgens are necessary to develop AGA and the androgen metabolism within target cells is of crucial importance. The literature, however, on normal and pathologic androgen metabolism (AM) is vast, and contradictory studies are a source of additional confusion. Here, some pivotal aspects of AM which are important for hair growth are reviewed.

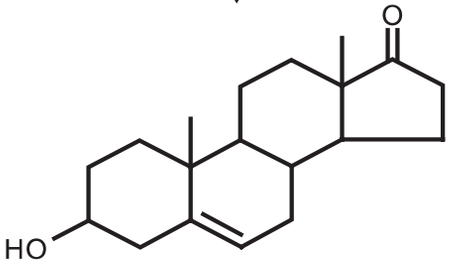
Androgen metabolism can be divided into glandular and extraglandular production, transport, target cell metabolism and cellular response. The synthesis of androgens is complex because it occurs in several organs, each of which has its own pecularities. The androgen metabolism of adrenals and gonads and the influence of the pituitary gland are beyond the scope of this review and are described in detail elsewhere.[21]

Androgen synthesis begins with cholesterol which is converted to pregnenolone. Following α-hydroxylation at the C17-position, the action of the enzyme C17-20 lyase cleaves distal carbon moieties, leaving a C-19 carbon steroid with a C-17 ketone in the distal ring. These '17-ketosteroids' make up a group of relatively weak androgens, such as dehydroepiandrosterone (DHEA), defined by their relatively low af-



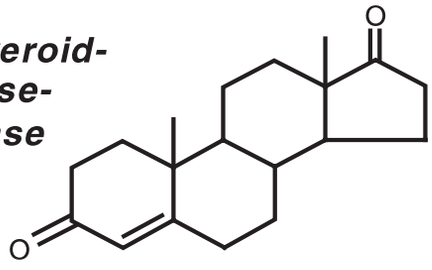
Dehydroepiandrosterone sulfate (DHEAS)

Steroid sulfatase



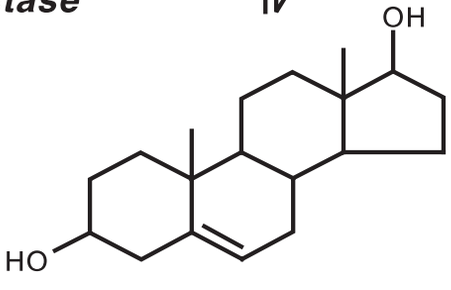
Dehydroepiandrosterone (DHEA)

3β-Hydroxysteroid-dehydrogenase-Δ 4-5 isomerase

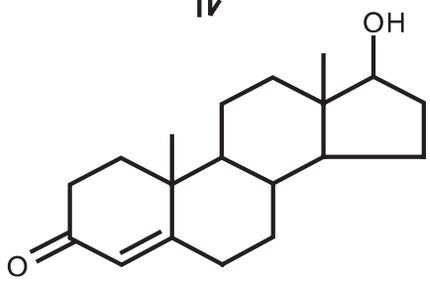


Androstenedione

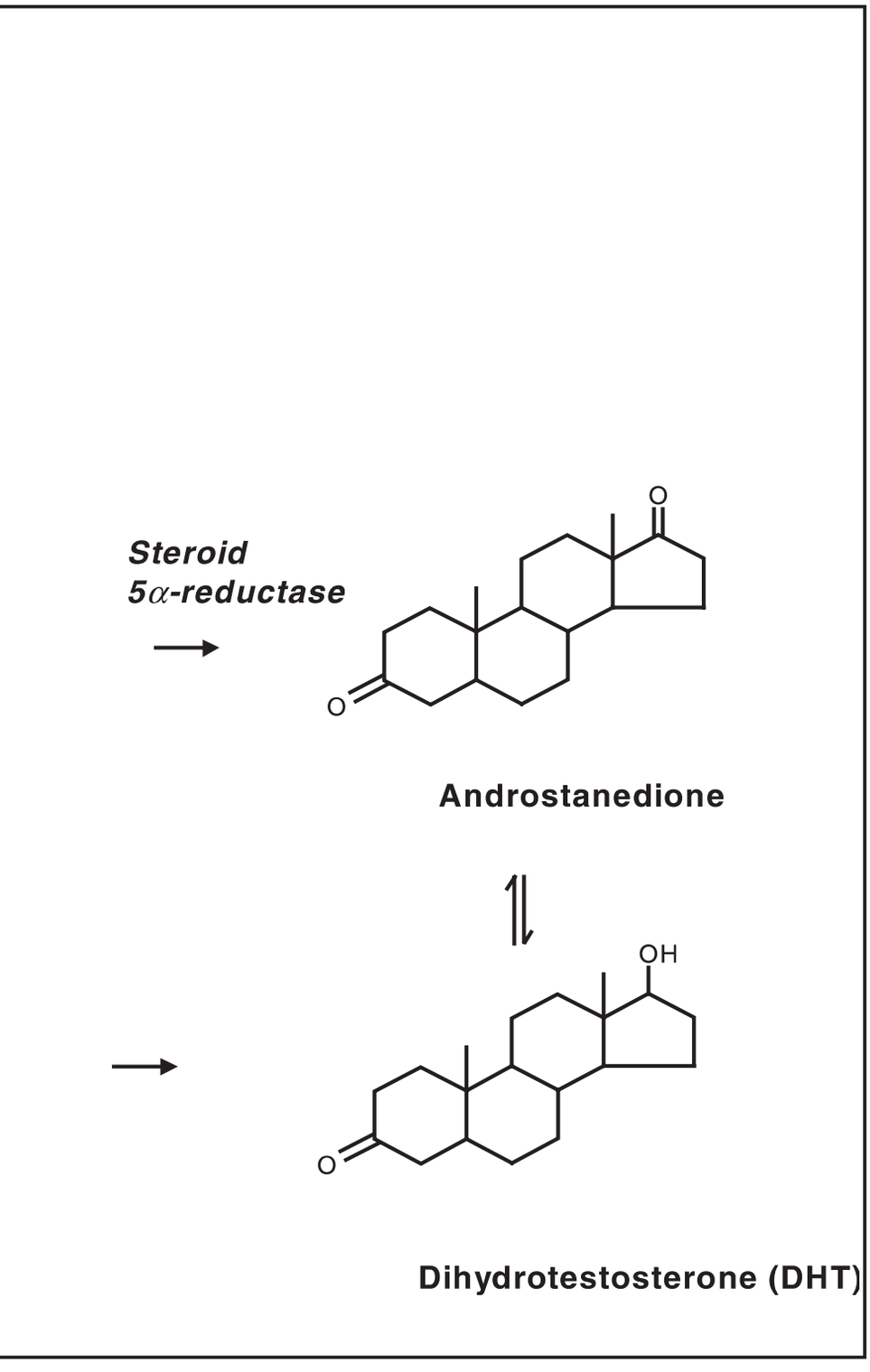
17β-Hydroxysteroid oxidoreductase



Androstenediol



Testosterone (T)



finity for the androgen receptor. Approximately 75% of DHEA and 95% of dehydroepiandrosterone sulphate (DHEA-S) is derived from the adrenal gland. These weak androgens can be enzymatically converted to more potent androgens such as testosterone, which is the major circulating androgen. In the hair follicle the principial pathways involved in the conversion of weak to more potent androgens

Figure 1 Androgen metabolic pathways.

are through activity of the enzymes 3β-hydroxysteroid dehydrogenase/$\Delta^{5\rightarrow4}$-isomerase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD). In most target organs testosterone can be further metabolized to DHT via the action of 5α-reductase (Fig. 1). The affinity of DHT to the androgen receptor is approximately fivefold higher than that of testosterone. Potent androgens such as testosterone or DHT can be removed by conversion to weaker androgens, or they can be metabolized via the aromatase to oestrogens, or they can be glucuronidated to form androgen conjugates that are more rapidly cleared from the circulation.

Some target tissues show enhanced AM and androgen sensitivity.[21] Circulating DHEA-S may be more rapidly metabolized to DHEA via steroid sulphatase. In turn DHEA may be more rapidly converted to androstenedione if increased 3β-HSD activity is present. Androstenedione may be converted to testosterone if 17β-HSD activity is present. If target cells convert weak androgens at an accelerated pace, then there will be enhanced conversion of testosterone to DHT. Another reason for increased sensitivity of a target to androgens is believed to involve an increase in the number of androgen receptors.

Only a small fraction of androgens exist as free steroids in the circulation, with an equilibrium between free androgen hormone and protein-bound androgens. The most important protein for androgen binding is sex-hormone binding globulin (SHBG). Approximately 70% of testosterone is bound to SHBG, 19% to albumin and only the remainder is unbound. Whether the bound fractions are still metabolically active is a matter of controversy, but binding of androgens to SHBG is an important factor in AM because it acts somehow as a sink for circulating testosterone.

Like all steroid hormones, androgens exert their effects by binding to an intracellular receptor, the androgen receptor. Binding of androgens to their androgen receptor leads to conformational change of the AR-androgen complex (ARAC) which is then transported into the nucleus where it can bind to regions of DNA that have distinctive binding sites known as androgen-responsive elements (ARE). A wide variety of proteins have this ARE encoded in their DNA. In this way androgens are able to modulate the transcription of various genes, that may be activated or suppressed.

In summary, the AM is highly complex and can be tuned at various points, e.g. the amount of weak androgens present

for conversion to more potent androgens, the repertoire of metabolizing enzymes present in target cells, the ratio of conversion and backconversion, the concentration of SHBG in the serum, the affinity of androgens to the androgen receptor, etc. Furthermore, most target organs differ in their repertoire of metabolizing enzymes and this repertoire is different in men and women. Moreover, many metabolizing enzymes have isoenzymes with different tissue distribution, substrate affinity and enzymatic kinetics.

The principal elements of androgen metabolism

Androgen-dependent processes are not the result of the summation of the activity of individual metabolites, but are solely due to the binding of DHT and translocation of the receptor to the nucleus. This concept has been discussed for the development of benign prostate hyperplasia[22] and is most likely valid for AGA as well. Therefore DHTdependent cell functions will only be initiated or amplified if:

- enough weak androgens are present for conversion;
- more potent androgens are formed via the action of 5α-reductase;
- the enzymatic activity of androgen inactivating enzymes

is low, e.g. aromatase;
- conversion of weaker steroids to DHT takes places by, e.g. 3β-HSD or oxidative 3α-HSD;
- functionally active androgen receptors are present in high numbers.

That this simplified concept is valid is nicely illustrated by mutations of androgen metabolizing genes, where often a lack of potent androgens leads to disturbed masculinization or to intersexuality.

Lessons to be learned from steroidogenic enzyme mutations

The synthesis and regulation of steroidogenic enzymes requires an orchestrated expression of biosynthetic enzymes in various tissues. Deficiency of one of these enzymes results in disturbed synthesis of one or more classes of hormones. Here, some genetic diseases affecting male sexual development and hair growth and the potential localization of these enzymes with the hair follicle will be described and their role during the pathogenesis of AGA will be briefly discussed.

Steroid sulphatase

The skin is able to synthesize active androgens, such as DHT, from the systemic precursor DHEA-S. The first step in

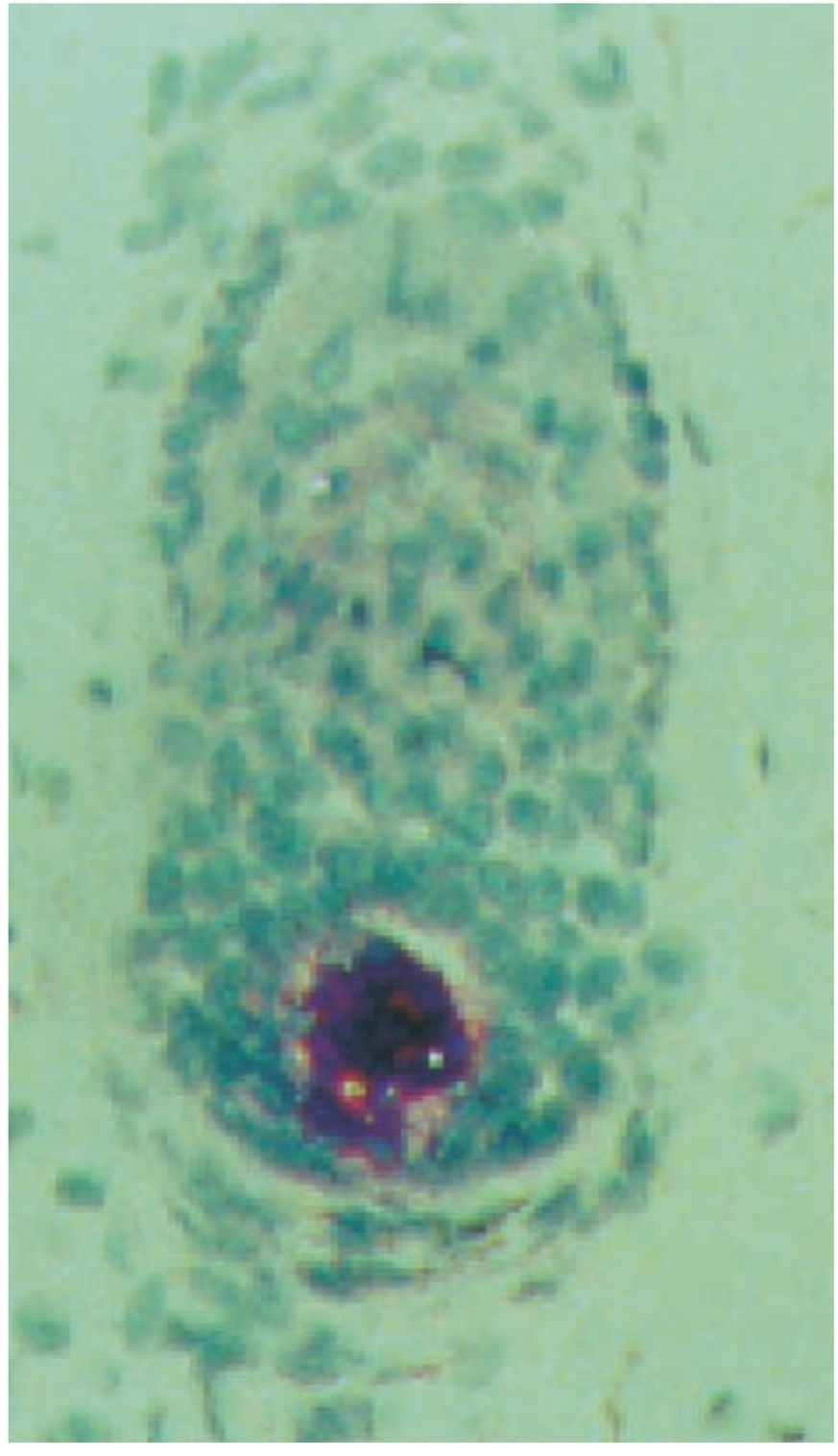


Figure 2 Steroid sulphatase immunoreactivity in human hair follicles: Immunohistochemical analysis using specific anti-steroid sulphatase antibody has been performed on sections of normal human skin. The steroid sulphatase immunoreactivity appears as a red stain. All sections were counterstained with haematoxylin. In this example a strong immunoreactivity in the dermal papilla can be seen in a human vellus hair (· 430).

this pathway is the desulphatation of DHEA-S by the enzyme steroid sulfatase. Because DHEA is further metabolized to androstendione, testosterone, and in special target tissues eventually to DHT, steroid sulfatase is an important enzyme for the conversion of the weak adrenal androgens to more potent androgens in the periphery. DHEA-S is believed to maintain axillary hair and is thought to be involved in the pathogenesis of hirsutism in women.[23] However, in women an excess of DHEA-S or its steroid sulfatase metabolite is believed to be involved in several androgen-dependent processes such as acne and AGA. Remarkably, DHEA-S and DHEA plasma levels seem to correlate with balding in young men indicating that steroid sulfatase may play a role in the pathogenesis of AGA.[24] Today the conversion of DHEA-S to DHEA by human hair follicles is well documented,[25] and recently we were able to show that steroid sulphatase is located mainly within the dermal papilla (Fig. 2).[26]

3β-hydroxysteroid dehydrogenase/$\Delta^{5\rightarrow4,}$-isomerase

The 3β-HSD isoenzymes catalyse an obligatory step in the biosynthesis of androgens, oestrogens, mineralocorticoids and glucocorticoids. These steroids play a crucial role in the differentiation, development, growth, and physiological

function of most human tissues. The enzyme is expressed in the adrenal cortex and in steroidogenic cells of the gonads, as well as in many other tissues such as the liver and kidney. The two 3β-HSD isoforms are expressed in a tissue-specific manner involving separate mechanisms of regulation. The structures of several cDNAs encoding 3β-HSD isoenzymes have been characterized in humans and other vertebrate species: human types I and II (Table 2); macaque; bovine; rat types I, II, III, and IV; mouse types I, II, III, IV, V and VI; hamster types I, II, and III.

The importance of the 3β-HSD in male steroid hormone physiology is underscored by a genetically determined deficiency that is transmitted as an autosomal recessive trait and

Table 2 Characteristics of 3β-HSD isoenzymes.

Characteristics	Type 1 3β-HSD	Type 2 3β-HSD
Size (amino acids)	372	371
Chromosome localization	1p 13.1	1p 13.1
Gene (exons)	4	4

is characterized by varying degrees of salt wasting. Foetal testicular 3β-HSD deficiency causes undervirilized male genitalia (pseudohermaphroditism); women exhibit either normal sexual differentiation or mild virilization. At least 24 mutations have been identified in 25 distinct families with 3β-HSD deficiencies, leading to slightly different clinical phenotypes. All mutations were detected in the type II 3β-HSD gene. No mutation was detected in the type I 3β-HSD gene, which is expressed in peripheral tissues. Whether hair growth is affected in these individuals has so far not been investigated, but because of the importance of 3β-HSD in AM and increased activity in AGA this question warrants further investigations. Plucked hair follicles (without sebaceous gland) ex vivo also exhibit marked 3β-HSD activity and we were able to detect this enzyme mainly within the dermal papilla of anagen hair follicles.[26]

17β-hydroxysteroid dehydrogenases

Isoenzymes of 17β-HSD regulate levels of bioactive androgens and oestrogens in a variety of tissues. At present five isoenzymes of 17β-HSD that differ in tissue expression and requirements for cofactors such as NADPH for type III 17β-HSD, and NAD(+) for type 2 17β-HSD are known (Table 3).

The importance of the type 3 enzyme in male steroid hormone physiology is underscored by the genetic disease 17β-HSD deficiency. Mutations in the type 3 17β-HSD gene impair the formation of testosterone in the foetal testis and give rise to genetic males with normal male Wolffian duct structures but female external genitalia very similar to the abnormalities seen in 5α-reducatase deficiency. These individuals are usually reared as females, but at puberty there is a striking rise in testosterone and they change their social sex. To date, more than 18 recessive mutations have been identified, giving rise to different clinical phenotypes. To our knowledge the presence or absence of AGA has so far not been investi-

Table 3 Characteristics of 17β-HSD.

Characteristics	Type 1 17β-HSD	Type 2 17β-HSD
Size (amino acids)	327	387
Gene (exons)	6	5
Chromosome	17q21	16q24
Cofactor preference	NADPH	NAD +
Catalytic preference	Reduction	Oxidation

gated. However, potential significance of 17β-HSD isoenzymes in AGA is underscored by observations of Hodgins et al.[27] who plucked hair follicles from young adults not yet expressing AGA, but with a strong family history of baldness, and found two populations, one with high 17β-HSD activity and one with low enzyme activity. Therefore, linkage of the genes encoding the 17β-HSD isoenzymes and AGA warrants further investigation. Very early it was shown that plucked human hair follicles or hair follicles from stump-tailed macaques express considerable 17β-HSD activity because the principle metabolite of testosterone is androstenedione (Fig. 1). The isoenzyme-specific expression pattern in different

Type 3 17β-HSD	Type 4 17β-HSD	Type 5 17β-HSD
310	736	323
11	–	9
9q22	–	10p14,15
NADPH	NAD +	NADPH
Reduction	Oxidation	Reduction

parts of the hair follicle has so far not been investigated in detail primarily because of technical problems. Only one study described type 1 and 2 17β-HSD in the epithelial parts of the hair follicle. The authors did not find mRNA for 17β-HSD in the dermal papilla. However, at the protein level, this enzyme is metabolically active within the dermal papilla of anagen hair follicles.[28]

5α-reductases

The microsomal enzyme steroid 5α-reductase is responsible for the conversion of testosterone into the more potent androgen DHT and the conversion of androstenedione to 5α-androstanedione (Fig. 1). 5α-reductase deficiency is a rare autosomal recessive trait that was first described by Nowakowski and Lenz[29] but without aetiological characterization which was not possible at that time. In 1974 it became clear that these individuals lack functional 5α-reductase and today we know that the type 2 5α-reductase is lacking.[30–32] Several mutations of the gene that encodes type 2 5α-reductase have been described, but not every mutation will result in complete deficiency of the enzyme. Therefore, the clinical presentation of patients with 5α-reductase deficiency varies considerably. In typical cases, a 46, XY male who has testes, normal plasma

testosterone and low DHT levels are observed. At birth a male ejaculatory system that terminates in a blind-ending vagina can be recognized together with a microphallus and a non-fused scrotum and maldescended testes. Therefore, these individuals display a female phenotype and are usually raised as girls. However, many affected individuals who were raised as females undergo a dramatic change of social sex at the time of expected puberty. They will have spermiogenesis, ejaculations and male-type sex drive. Interestingly, no or minimal beard growth or AGA is seen in these men. These observations together with the finding that both humans

Table 4 Characteristics of 5α-R isoenzymes.

Characteristics	Type 1 5α-reductase	Type 2 5α-reductase
Size (amino acids)	259	254
Molecular weight	29 kDa	28 kDa
pH-optima in vitro	6–9	5,5
Chromosome localization	5p15	2p23
Gene (exons)	5	5

and stump-tailed macaques have beard and frontal scalp hair follicles with higher 5α-reductase activity than hair follicles from the occiput[12,33] indicates that the type 2 5α-reductase is involved in the pathogenesis of androgen-dependent hair growth. The inhibition of this isoenzyme is therefore a rational approach for treatment.

Two distinct 5α-reductase isoforms have been cloned. Subsequently it has been shown that these isoenzymes have distinct molecular, biochemical and tissue expression characteristics (Table 4). In humans mutations in the gene encoding type 1 5α-reductase have not been reported. In mice, however, a mutation in this gene will cause early foetal death because of oestrogen excess in utero.

Special attention has been paid to the dermal papilla and several authors have tried to localize both isoenzymes within the dermal papilla. Some authors were unable to find considerable 5α-reductase activity in occipital scalp dermal papilla, whereas others found this enzyme in beard and occipital scalp dermal papilla.[34,35] Recently, we were able to show that the main metabolic activity of type 2 5α-reductase can be detected in intact occipital scalp and beard dermal papilla.[36] Provided the dermal papilla plays a crucial role during

androgenmediated processes on the hair follicle, our results suggest that the dermal papilla might amplify testosterone-driven responses in the human hair follicle via the action of type 2 5α-reductase.

Oxidative 3α-hydroxysteroid dehydrogenases

Once formed, DHT is further inactivated via 3α-HSDs to the weaker androgens androsterone and androstanediol. In theory the back conversion of these weak steroids to DHT via oxidative 3α-HSD may promote DHTdependent hair loss. Recently we were able to demonstrate that such metabolism is present in the dermal papilla of occipital and beard hair follicles and theoretically any drug that is able to block this process might be beneficial for AGA (unpublished data).

Aromatase

The cytochrome P450 aromatase (P450arom) enzyme is required for bioconversion of androgens to oestrogens. Only a single human gene encoding aromatase P450 (CYP19) has been isolated. Mutations in the CYP19 gene do rarely occur and result in aromatase deficiency. Girls show pseudohermaphrodism at birth which sometimes is corrected by surgical repair of the external genitalia, including a clitoridectomy. At puberty, they develop virilization, pubertal failure with

no signs of oestrogen actions, hypergonadotropic hypogonadism, polycystic ovaries on pelvic sonography, and tall stature. Males are tall with eunuchoid skeletal proportions. Their bone age is retarded and osteopenia can be observed, indicating that oestrogens are important for bone development. At puberty affected females will develop hirsutism due to an androgen excess and in theory females and males might develop early onset AGA. However, this question has not been reported or investigated.

Women tend to develop AGA later in life and in a milder form than men. With the decline of serum oestrogens during menopause many women show an accelerated progression of AGA. Oestrogens may play a protective role against the development of AGA, because pregnant women with high levels of oestrogens show a prolonged anagen phase, but lose their hair again post-partum. Recently it has been shown that hair follicles from women with AGA express more aromatase activity as compared with male-derived hair follicles,[37] and interestingly those women taking aromatase inhibitors tend to develop AGA rather rapidly.[38] These circumstantial lines of evidence indicate a role of aromatase in the pathogenesis of AGA. In order to unravel the pathways of oestra-

diol-mediated effects on the hair follicles, we measured aromatase activity in isolated intact human occipital hair follicles by incubating hair follicles and found aromatase to be expressed mainly within the root sheaths of the hair follicle. However, some cells of the stalk region of the dermal papilla also stained for aromatase. We also noticed that, in comparison with controls oestradiol-incubated female hair follicles showed a concentration- and time-dependent increase of aromatase activity. We concluded that an increased conversion of testosterone to 17β-oestradiol, and androstendione to oestrone, takes place in hair follicles derived from the occiput under the influence of estradiol. In theory, this pathway may diminish the amount of intrafollicular testosterone available for conversion to DHT.[39]

Interaction of DHT with AR and ARE

DHT is a pivotal trigger of androgen-mediated effects on the hair follicle and the principal signal transduction cascade: DHT- DHT/androgen receptor -ARE is similar in all hair follicles. However, DHT makes some hair follicles grow whereas others will miniaturize. Something fundamentally different must be present in beard vs. frontal hair follicle target cells. At present this paradox is not understood but an ac-

cumulating body of evidence indicates that the androgen receptor or distinct ARE might be involved in this process.

Androgen receptor

Without functionally active androgen receptors a genetically male foetus will not undergo normal male development in utero and a phenotypically female child is born. This is demonstrated by the various forms of androgen insensitivity syndrome (AIS). Several mutations in the gene for the androgen receptor may lead to AIS, but not every mutation will result in complete absence of functional androgen receptors. Interestingly complete AIS (grade 8) is characterized by a female phenotype despite a male genotype and lack of pubic hair, whereas incomplete AIS (grade 7) pubic hair is present. To our knowledge defined mutations of the androgen receptor and their effect on AGA have not been systematically looked for, but it is conceivable that some mutations may prevent balding. The literature on the localization of androgen receptors within the hair follicles is controversial because different antibodies located the androgen receptors in different compartments of the hair follicles. One group did not find androgen receptors in the dermal papilla and the hair follicle epithelium, whereas other groups described

an intense staining for androgen receptors in the sebaceous gland, the dermal sheath and the outer root sheath of the hair follicles, as well as the dermal papilla in hair follicle of stump-tailed macaques, which is in accordance with results obtained in humans.[40] The different results were explained by different antibodies and different fixation techniques. Attempts to show differ- ences in the quantitative concentrations of androgen receptors in bald vs. hairy scalp have yielded conflicting results. In vitro it has been shown that dermal papilla cells from an androgen sensitive body site contain more androgen receptors than dermal papilla cells from androgen-insensitive hair follicles.[40]

Androgen-responsive genes

After forming the DHT/androgen receptor complex and transport into the nucleus, this complex will bind to distinct DNA binding sites of androgen-susceptible genes. In the prostate DHT affects genes such as the prostate steroid-binding protein or testosteronerepressed prostate message (TRPM). By means of differential RT–PCR, androgen-responsive genes have been found in human foreskin fibroblasts and prostate smooth muscle cells. For the hair follicle, however, such mechanisms are almost unknown. This is mainly

due to technical problems, because of the small size of single hair follicles and their intrafollicular compartments. In the prostate DHT induces 5α-reductase activity in an autocrine manner. Transforming growth factor-β1 has been shown to inhibit 5α-reductase in genital skin fibroblasts.[41] Conflicting results exist for insulin-like growth factor (IGF-1). This protein has been shown to induce 5α-reductase activity,[42] but other groups were unable to confirm these data.[43] In vitro, dermal papilla cells respond differently to exogenous testosterone, DHT, or estradiol.[44] Proliferation is inhibited by testosterone and DHT but not by estradiol. The physiologic significance of these data is difficult to interpret because in vivo dermal papilla cells do not proliferate. However, dermal papilla cells in vitro retain some of their in vivo parameters, and it has been shown that nexin-1 is not only present in the anagen hair bulb,[45] but also that this gene is regulated by androgens[46] which might indicate a role during the pathogenesis of AGA. Androgen-dependent hair follicles secrete soluble factors in response to testosterone such as IGF-1 that stimulate the growth of follicular epithelial cells[47] or inhibit the growth of complete hair follicles.[48] Other groups have reported on the secretion of stem cell factor[49,50] upon stimu-

lation with testosterone. Whether this is of pathophysiological importance in AGA is not yet known. Recently the role of caspases have been stressed during the process of hair follicle miniaturization.[51]

Conclusions and future perspectives

AGA can be defined as a DHT-dependent process with continuous miniaturization of sensitive hair follicles. The type 2 5α-reducatase plays a central role through the intrafollicular conversion of testosterone to DHT. So far the predisposing genes for AGA are unknown and we do not understand the molecular steps involved in androgen-dependent beard growth vs. androgen-dependent hair loss. However, with the cloning of the entire human genome, we may have new resources to explore the etiopathogenesis of AGA in more detail. With increasing knowledge of the follicular repertoire of isoenzymes involved in androgen metabolism, new nontoxic and selective inhibitors may turn out to be fruitful therapeutic modalities. The same is true for selective androgen receptor blockers such as RU 58841,[48] or those drugs interfering with the DHT-dependent signal transduction cascade in hair follicles. We may also remember a statement by

Van Scott and Ekel that was made more than 40 years ago: If the assumption is made that the size of a hair depends on the size of its papilla, a search for factors controlling the size of the papilla would seem to be appropriate in further investigations of male type baldness.[20] It will be fascinating to see what will be launched for the treatment of AGA in the next few years. We will soon know the underlying genes, and this may give us the opportunity of a gene therapy targeting the hair follicle.

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Correspondence: R. Hoffmann, Zentrum für Hautkrankheiten, Philipps-Universität, Deutschhausstraße 9, D-35033 Marburg, Germany. Tel.: +49 6421 286 2944. Fax: +49 6421 286 5728. E-mail: rolf.hoffmann@mailer.uni-marburg.de

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COMMENTARY: Hair Follicle Biology, the Sebaceous Gland, and Scarring Alopecias

OF ALL structures in or on the mammalian body, the hair follicle has one of the most complex functions. It must produce, on the skin surface during a long period, a multicellular product, the hair shaft, and yet preserve in the deep dermis an "epithelial finger" that produces the shaft. The cells making up the shaft contain the machinery to produce strong cytoskeletal and cellular adhesions and to be molded by the inner root sheath. The combined hair shaft–inner root sheath structure moves outward as a unit, sliding along a slippage plane provided by the innermost layer of the outer root sheath. The outer root sheath remains behind and intact. The shaft is liberated from the sheath at a level just below the sebaceous duct, and it exits the pilary canal as a sheath-free hair fiber.

Early researchers recognized that removal of the sheath from the shaft was a crucial event in hair processing, without which the shaft could not easily exit the skin. Because sheath dissolution occurs just below the sebaceous duct, it was postulated that the sebaceous gland, or the region of the follicle at this level, might be responsible for its separation.[1-3] Experimental evidence for this conclusion was found serendipitously during a study of sheep hair follicle growth in culture,[4] in which it was demonstrated that the sebaceous gland (or the midfollicular region) was necessary for the dissolution of the sheath from the shaft. Subsequently, this phenomenon was corroborated in human[4,5] and horse follicles.[6] These findings implicated an important role for sebaceous glands in skin besides synthesizing and liberating its emollient. What the active agent might be in sebum that causes sheath dissociation is not known but is under current investigation.

We tested the idea that the sebaceous gland is central to hair biology by asking the question, What happens to hair follicles in an animal that has defective sebaceous glands? This question led us to the asebia mouse mutant,[7] a mouse that forms hair follicles with markedly hypoplastic sebaceous

glands. After the first hair cycle, this mouse has progressive hair follicle loss and striking scarring alopecia. In a detailed histological study,[8] we found that cornified plugs in the hair canal apparently impede hair shaft egress. The histological findings suggested that the shaft, prevented from exiting the pilary canal, pushes the follicle in reverse toward the dermis, giving rise to the abnormally long and deep anagen structures seen. In support of the idea of distal pilary canal resistance, we could demonstrate that the shaft often perforated the bulb (the proximal follicle) in association with follicle-destructive chronic inflammatory and foreign body reactions. In the absence of normal sebaceous gland function, then, not only is sheath inadequately separated from shaft, as we found experimentally, but there is also follicle destruction and dermal scarring.

Although chronic, progressive alopecia develops in all 3 spontaneous asebia mutations (ab, abJ, and ab2J), scarring alopecia in association with sebaceous gland pathology is not unique to the asebia mouse mutant. Images published of the bare skin mouse mutant (mapping to mouse chromosome 11) indicate that the inner root sheath is retained much higher into the pilary canal (infundibulum) than normal;

moreover, in this mouse mutant, sebaceous glands are morphologically abnormal.[9] Harlequin ichthyosis mice also show alopecia associated with thick epidermal scales.[10] These mice have hypoplastic sebaceous glands and manifest large and compact cornified plugs within their pilary canal (infundibulum). These mice die at 10 to 12 days of age, prior to the anticipated onset of a true scarring alopecia.

Observations with these mouse mutants led us to reconsider the role of the sebaceous gland in human scarring alopecias. Most of the inflammatory, scarring alopecias in humans show an intense inflammatory cell reaction about the follicle at the level of the isthmus and upper infundibulum. Since tissue destruction in this region might include the "bulge" zone, the putative site of follicular stem cells has been implicated as the mechanism for follicular destruction seen in these disorders.[11] In fact, in most if not all forms of the "classic" inflammatory, scarring alopecias (alopecia mucinosa, lupus erythematosus, lichen planopilaris, pseudopelade of Brocq, follicular degeneration syndrome, and scleroderma), the sebaceous glands appear to be a common victim during the early course of the disease. Similar observations have been made with sebaceous adenitis with hyperkerato-

sis in dogs and cats.[12-14] It is generally believed that the sebaceous gland is destroyed before the follicle — the sebaceous gland is lost first. The mechanism for sebaceous gland loss may be multifold. One such mechanism is that sensitized lymphocytes might attack the proliferating basal cells of the sebaceous gland as observed in graft-vs-host disease.[15] As a representative example of a scarring alopecia, the earliest changes in the follicular degeneration syndrome consist of sebaceous gland loss in the presence of a relatively intact hair-forming epithelium.[16] The hair-forming epithelium is eventually destroyed late in the disease course, but this appears as a sequel to the sebaceous gland loss.

Our studies and those of the literature do not explain why the sebaceous gland is lost in the early phases of the human scarring alopecias or even if there is a cause-and-effect relationship. In the case of the mouse, however, the data are compelling. The asebia mouse has shown us that the mutation of a gene unique to sebaceous gland function leads to a scarring alopecia.[9,17]

In conclusion, the hypothesis we are proposing is that many of the scarring alopecias, which have proven to be mechanistically obscure, are based on primary sebaceous

gland pathological features. In vitro studies indicate an important role of the sebaceous gland–isthmus in dissociating the internal root sheath from the shaft. Several mouse mutants showing pathologic features of the sebaceous gland manifest keratinous follicular plugging. If inadequate sebaceous gland function could lead to hair follicle destruction, perhaps we should reassess our treatment protocols and give more attention to the superficial-lying sebaceous gland than to the deeper-lying anagen follicle.

Kurt S. Stenn, MD, Skin Biology Research Center, Johnson & Johnson Consumer Products, Inc, 199 Grandview Rd, Skillman, NJ 08558 • John P. Sundberg, DVM, PhD, Bar Harbor, Me • COL Leonard C. Sperling, MC, USA, Bethesda, Md

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