Adenosine stimulates growth of dermal papilla and lengthens the anagen phase by increasing the cysteine level via fibroblast growth factors 2 and 7 in an organ culture of mouse vibrissae hair follicles

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Abstract. Hair regression and balding are distressing concerns for an increasing number of people due to changes in lifestyle and serious nutritional imbalances. Therapies for treatment of hair loss are needed. Among potential therapeutics, adenosine has been suggested as a potent regulator of hair growth. In this study, we investigated the effects of adenosine on hair follicles and dermal papilla (DP) cells, and the mechanism underlying the action of adenosine. Hair follicles are organs, including DP cells, that are responsible for the production of hair fibers by inducing and maintaining the hair growth phase (anagen). In a culture of DP cells *in vitro*, adenosine stimulated proliferation of DP cells by increasing thymidine uptake. Subsequently, adenosine activated and elongated the anagen phase by increasing the uptake of radiolabeled cysteine in an organ culture of mouse vibrissae hair follicles. We also confirmed that adenosine promoted the expression of several growth factors that are responsible for hair growth, including fibroblast growth factors (FGF)-7, FGF-2, insulin-like growth factor (IGF)-1, and vascular endothelial growth factor (VEGF) in a cDNA microarray with semi-quantitative RT-PCR. Transcriptional activation of β-catenin in DP cells was increased by adenosine in a luciferase assay. β-catenin is a co-activator of Wnt/β-catenin signaling that induces morphogenesis and differentiation of hair follicles and also acts to transactivate downstream signaling pathways, including the ERK pathway. Using Western blotting, we found that adenosine stimulated phosphorylation of ERK, CREB and AKT. These results suggest that adenosine stimulates growth of hair follicles by triggering the expression of growth factors and β-catenin, and by inducing their downstream target signaling pathways.

Introduction

Hair growth is a complex and cyclically controlled process that is categorized by a finite period of hair fiber production (anagen), a brief regression phase (catagen), and a resting period (telogen). Although the precise mechanism regulating the hair growth cycle has not been fully characterized, it is well known that both the continuation and the transition of each phase result from elaborate interactions between mesenchymal dermal papilla and adjacent follicular epithelium, the matrix, and the outer root sheath (ORS) (1). In particular, mesenchyme-derived dermal papilla (DP) cells, located at the base of the hair follicle and surrounded by the matrix, have the potential to induce hair follicle formation when implanted into the lower part of an amputated hair follicle *in vivo* and to retain their hair follicle inductive properties when cultured *in vitro* (2). A number of signals from DP cells may directly influence the surrounding matrix cells and ORS cells in the bulge area, and then regulate the hair growth cycle (3). These signals include fibroblast growth factors (FGF), insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), transforming growth factor-β (TGF-β), Wnt/β-catenin, bone morphogenetic proteins, and sonic hedgehog (4-7).

Adenosine is known to have various physiological functions, including inhibition of lipolysis, vasodilation, and protection against ischemic damage. These functions are mediated via the up- or down-regulation of cAMP, inositol triphosphate (IP3), and IP3/diacylglycerol via the G protein coupling receptors of the four androgen receptor subtypes A1, A2a, A2b and A3 (8). Adenosine has also been reported to be a potent regulator of hair growth in dermal papilla cells. Li *et al* (9) proposed an adenosine mediated mechanism for minoxidil-induced vascular endothelial growth factor (VEGF) production by the signaling pathway. Iino *et al* (10) reported that adenosine stimulates FGF-7 gene expression. However, the potential effects of adenosine on hair growth have not been fully elucidated.

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In this study, we examined the effects of adenosine on hair follicles and DP cells and the underlying mechanism of adenosine action. In a culture of DP cells *in vitro*, the effect of adenosine was determined on proliferation of DP cells using a thymidine uptake assay. We also examined the effect of adenosine on the transition from the anagen to the telogen pahses, and growth stimulation using a radiolabeled cysteine assay in an organ culture of mouse vibrissae hair follicles. We further investigated the effect of adenosine on gene expressions of several growth factors that are responsible for hair growth, including FGF-7, FGF-2, IGF-1 and VEGF using a cDNA microarray and semi-quantitative RT-PCR.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA), Williams E medium (Gibco-BRL), and fetal calf serum (FCS; Gibco-BRL) were used for mouse vibrissa organ and human hair dermal papilla (DP) cell cultures. For the $[^{35}S]$-cysteine uptake and the $[^{3}H]$-thymidine incorporation assays, $[^{35}S]$-cysteine and $[^{3}H]$-thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK, USA) were incubated with mouse vibrissa hair follicles and DP cells, respectively. C57BL/6 mice were supplied by Dae-Han Biolink (Eumsung, Chungbuk, Korea). Adenosine was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Organ culture of hair follicles and [^{35}S]-cysteine uptake assay. A mouse vibrissa organ culture was conducted according to a previously reported method (11). Anagen phase follicles were carefully dissected from the upper lip pad of 6-week-old female C57BL/6 mice and maintained in Williams E medium (Gibco-BRL) supplemented with 10 ng/ml of insulin (Gibco-BRL) and 10 ng/ml of hydrocortisone (Gibco-BRL). Follicles were incubated in the presence of 1 μCi of $[^{35}S]$-cysteine and 400 μM adenosine (Sigma-Aldrich) for 3 days. After washing twice in phosphate-buffered saline (PBS), follicles were lysed using a tissue solubilizer (Packard Instrument Company, Meriden, CT, USA) at 56°C for 1 h, then radioactivity was measured using a liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA, USA).

Culture of normal and SV40-transformed human DP cells. Human hair DP cells were cultured according to the method of Messenger (12) with a slight modification. These cells were isolated under a stereomicroscope using watchmaker's forceps and transferred into DMEM (Gibco-BRL) supplemented with 10% FCS (Gibco-BRL). DP cells were grown normally for 5-7 days after explanting. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Third or fourth passage DP cells were used in this study. Immortalization of the DP (SV-DP) cells was achieved by infection with a retrovirus carrying the SV40 T antigen and the neomycin resistance gene, followed by G418 (Gibco-BRL) selection (13). The retroviral vector pLXIN-SV40T was stably transfected into recombinant retrovirus packaging cell line PT67 cells (Clontech Laboratories, Inc., Mountain View, CA, USA). The retrovirus-containing medium was collected, filtered through a 0.22 μm low protein binding filter (Millipore Corporation, Billerica, MA, USA), and then transferred onto primary cultured DP cells. After infection overnight, the retrovirus-containing medium was replaced with fresh medium, followed by further incubation for 2 days. Transfectants were selected in G418-containing medium (1 mg/ml; Gibco-BRL) for 4 weeks.

[^{3}H]-thymidine incorporation assay. For a $[^{3}H]$-thymidine incorporation assay, DP cells were plated at $2x10^4$ cells/well of 6-well plates and grown in DMEM (Gibco-BRL) supplemented with 10% FCS (Gibco-BRL) for 24 h. After washing twice in PBS, cells received DMEM without FCS, 1 μCi of $[^{3}H]$-thymidine, and 10, 100 or 200 μM of adenosine. Following incubation for 48 h, cells were washed twice in PBS and then lysed using 0.1 N NaOH and 1% sodium dodecyl sulfate (SDS). Radioactivity was then measured using a liquid scintillation counter.

Total-RNA extraction and cDNA microarray analysis. Total-RNA was extracted from cells using a TRIzol reagent kit (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. For microarray analysis, a 22K Human Genome Array (Genomictree Inc., Daejeon, Korea) was used. Total-RNA (100 μg) was reverse-transcribed in the presence of Cy3 and/or Cy5 monoreactive dye (Amersham Pharmacia Biotech) using a Superscript cDNA synthesis system (Gibco-BRL). After hybridization, microarray scanning and data normalization were performed using a GenePix 4000B scanner and GenePix Pro 3.0 software (Axon Instruments, Inc., Union City, CA, USA). Differentially expressed genes were selected based on a fold-change and the Welch's t-test.

Reverse transcription-polymerase chain reaction (RT-PCR). DP cells were grown on 100-mm tissue culture dishes to ~80% confluency in DMEM (Gibco-BRL) supplemented with 10% FCS (Gibco-BRL). After washing twice in PBS, DP cells were cultured in a serum-free medium for 18 h. Cells were cultured for 6 h in FCS-free medium, together with 100 or 400 μM adenosine. After total-RNA extraction, 2 μg of total-RNAs was reverse-transcribed using M-MLV reverse transcriptase (ELPIS Biotech, Inc., Daejeon, Korea) in the presence of a random hexamer. Aliquots of the resultant RT mixtures were then subjected to PCR cycles as follows: 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min for 35 cycles for FGF-2 and IGF-1, and 30 cycles for FGF-7 and VEGF. Primers for amplifying the respective fragments are listed in Table I. After agarose gel electrophoresis, PCR products were quantified using a densitometer (Imagemaster; Amersham Pharmacia Biotech).

Western blot analysis. Cells were lysed in Proprep solution (iNtRON Biotechnology, Sungnam, Kyeonggido, Korea). After vigorous pipetting, extracts were centrifuged for 15 min at 13,000 rpm. The total protein content was measured using a Bradford protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples were run on SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and incubated with appropriate antibodies overnight at 4°C with gentle agitation. Blots were then incubated with peroxidase-conjugated secondary antibodies for 30 min at room temperature, and visualized using enhanced chemiluminescence (iNtRON Biotechnology). The following primary antibodies were used in this study: collagen type 1 α1, elastin, β-catenin and actin

Table I. Nucleotide sequences of primers.

Gene name		Sequences	Expected size (bp)
FGF-7	Sense:	5'-ACACACAACGGAGGGGAAAT-3'	552
	Antisense:	5'-GCCATAGGAAGAAAGTGGGC-3'	
FGF-2	Sense:	5'-TGACCAACATGGTGAAACCC-3'	398
	Antisense:	5'-AACTGATCCCAGGAGATGGC-3'	
IGF-1	Sense:	5'-GATTCTCCTGCCTCAGCCTC-3'	429
	Antisense:	5'-TAAGAGGGAACACATGGGCA-3'	
VEGF	Sense:	5'-CTACCTCCACCATGCCAAGT-3'	536
	Antisense:	5'-GCGAGTCTGTGTTTTTGCAG-3'	
G3PDH	Sense:	5'-GTCAGTGGTGGACCTGACCT-3'	420
	Antisense:	5'-AGGGGTCTACATGGCAACTG-3'	

(Santa Cruz Biotechnologies, Santa Cruz, CA, USA); total-p38 mitogen-activated protein kinase (MAPK), phospho-p38 MAPK, total-p42/44 extracellular signal-regulated kinase (ERK), phospho-p42/44 ERK, total-AKT, phospho-AKT, total-cAMP response element-binding (CREB), and phospho-CREB (Cell Signaling Technology, Beverly, MA, USA).

Luciferase assay. Replication-incompetent adenoviruses were created using the ViraPower™ adenovirus expression system (Invitrogen Life Technologies) according to the method provided by Invitrogen. Site-specific recombination between the entry vector and an adenoviral destination vector was achieved using LR clonase (Invitrogen Life Technologies). The resulting adenoviral expression vector was then transfected into 293A cells using Lipofectamine 2000 (Invitrogen Life Technologies). Cells were grown until an 80% cytopathic effect (CPE) was seen, then harvested for preparation of recombinant adenovirus. SV-DP cells at 50% confluency were co-transduced with the pTOPFLASH-luc reporter adenovirus carrying the TCF-binding consensus sequence followed by the luciferase gene, and incubated for 18 h. After replenishing with fresh medium, cells were treated with adenosine for 24 h, and then cellular extracts were prepared using a cell lysis buffer. The luciferase activitiy was determined using the Luciferase assay system (Promega Corporation, Madison, WI, USA) according to the recommended protocol of Promega. The luciferase activitiy indicates the transcriptional activation of β-catenin, which acts as a co-activator of TCF proteins to regulate gene expression.

Statistical analysis. Data from the [^{35}S]-cysteine uptake and [^{3}H]-thymidine incorporation assays were statistically evaluated using the Student's t-test. Statistical significance was set at $P<0.05$.

Results

Adenosine activates and elongates the anagen phase of mouse vibrissae hair follicles in vitro. We investigated the effects of adenosine on a mouse hair follicle culture. Isolated mouse vibrissa follicles were cultured for 3 days in the presence of [^{35}S]-cysteine and adenosine at the indicated concentrations. Treatment of mouse hair follicles with adenosine resulted in a decrease in the ratio of hair follicles with a catagen-like shape compared to control follicles (Fig. 1). After a 3-day incubation, most hair bulbs (92%) at the bottom of untreated follicles had degenerated. However, 37% of follicles treated with adenosine maintained an anagen-like morphology, although regression was observed to some extent (Fig. 1A and B).

In addition, adenosine increased the uptake of radiolabeled cysteine, which is a major amino acid in cysteine-rich keratins, the structural proteins of hair fibers (Fig. 1C) (14). Since [^{35}S]-cysteine uptake is correlated to hair follicle growth *in vitro* (15), these results suggest that adenosine produces a stimulating effect on hair growth.

Adenosine stimulates thymidine uptake in human hair dermal papilla cells. It has been previously reported that adenosine has a stimulatory effect on the proliferation of various cell types, including endothelial, chick astrocyte, and human astrocytoma cells (16). We investigated whether adenosine has the potential to stimulate the growth of DP cells using a [^{3}H]-thymidine uptake assay. When DP cells were treated serially with adenosine and incubated for 2 days, adenosine apparently increased tritium [^{3}H]-thymidine uptake by DP cells in a dose-dependent manner (Fig. 2).

Adenosine induces β-catenin in SV-DP cells. Since Wnt/β-catenin signaling has been proposed to function in hair follicle morphogenesis and differentiation, we investigated the effect of adenosine on the transcriptional activity of β-catenin in dermal papilla cells. SV-DP cells transduced with the pTOPFLASH-luc reporter adenovirus were treated with adenosine for 24 h. Adenosine treatment led to an increase in the transcriptional activity of β-catenin in a dose-dependent manner (Fig. 3).

Effects of adenosine on the FGF-2 and FGF-7 genes in cDNA microarray analysis. We performed DNA microarray analyses on a total of 29,016 genes from DP cells in the presence and absence of 100 µM adenosine for 6 h. Adenosine treatment induced a more than 2-fold increase in 1,305 genes, including FGF-7 (4.97±1.77 fold) and FGF-2 (3.13±0.85 fold) (Table II).
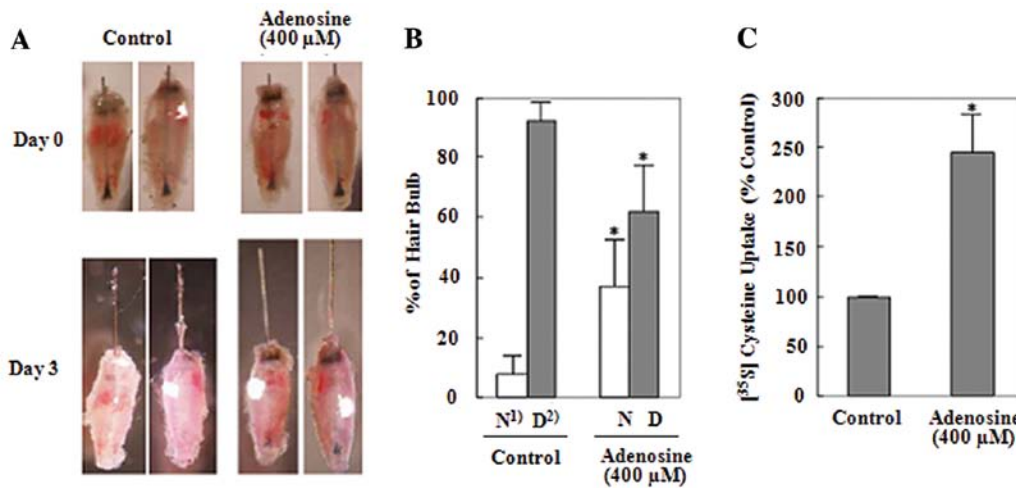
Figure 1. Effects of adenosine on a mouse hair follicle culture. Mouse vibrissa follicles were isolated and cultured in William's medium E with and without adenosine for 3 days. (A and B) Images of mouse vibrissa follicles at Days 0 and 3 cultured in the absence and presence of 400 μM adenosine. After a 3-day incubation, regression of the hair bulb was observed in most control hair follicles (93%). The hair bulb structure of more than one third (37%) of the follicles treated with adenosine was preserved (n=58). N[1], Normal hair follicles maintaining an anagen-like morphology; D[2], degenerated hair follicles in the regression phase. (C) After a 3-day incubation in the presence of [35S]-cysteine, follicles were lysed using tissue solubilizer and radioactivity was measured using a liquid scintillation counter. Adenosine increased the uptake of radiolabeled cysteine. The amount of [35S]-cysteine uptake was expressed as percentage of a control. The mean values ± SE are averages of triplicate measurements (*P<0.05 vs. control). N[1], Hair bulb maintained anagen phase morphology; D[2], degenerated hair bulbs.
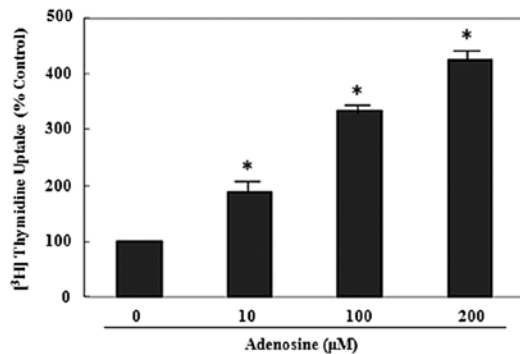

Figure 2. Effect of adenosine on growth of human hair dermal papilla (DP) cells cultured *in vitro*. DP cells were cultured in the presence of [3H]-thymidine for 2 days. Cells were lysed using 0.1 N NaOH, 1% SDS and radioactivity was measured using a liquid scintillation counter. The amount of [3H]-thymidine incorporation was expressed as percentage of a control. The mean values ± SE are averages of triplicate measurements (*P<0.05 vs. control).
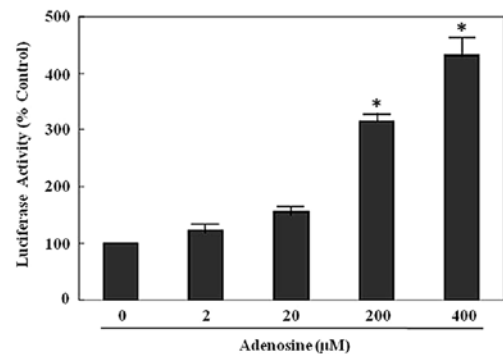
Figure 3. Effects of adenosine on the transcriptional activity of β-catenin in SV-DP cells. (A) SV-DP cells transduced with the pTOPFLASH-luc reporter adenovirus were treated with adenosine for 24 h, and cellular extracts were prepared. Transcriptional activities of β-catenin were determined using a luciferase assay and expressed as percentage of a control. The mean values ± SE are averages of triplicate measurements (*P<0.05 vs. control).

To confirm the cDNA microarray results, we performed semi-quantitative RT-PCR analysis. The expression levels of FGF-7 and FGF-2 were increased by adenosine in a dose-dependent manner (Fig. 4), indicating that the real-time PCR results are in agreement with the cDNA microarray results.

Effects of adenosine on the gene expression levels of growth factors in DP cells. To confirm the cDNA microarray results, we performed semi-quantitative RT-PCR analysis to investigate the effects of adenosine on expression of mRNA for several growth factors that are implicated in the regulation of hair growth. We measured the expression levels of IGF-1, VEGF, FGF-7 and FGF-2. When DP cells were treated with adenosine, the expression levels of FGF-7 and FGF-2 were increased by adenosine in a dose-dependent manner, in agreement with the cDNA microarray results shown in Fig. 4. In addition, the levels of IGF-1 and VEGF mRNA expression

also increased significantly at the time points indicated in the legend of Fig. 4.

Adenosine induces phosphorylation of ERK, CREB, and AKT. Since intracellular signaling cascades are implicated in the regulation of growth in a variety of cell types and tissues, we investigated the effects of adenosine on p42/44 ERK, CREB and AKT in SV-DP cells. Adenosine treatment led to phosphorylation of p42/44 ERK, CREB and AKT in a dose-dependent manner after 30 min (Fig. 5). These results suggest that adenosine affects intracellular signaling cascades, thereby influencing the growth of DP cells.

Discussion

The number of people who suffer from hair loss or hair thinning is increasing. Thus, it is important to develop thera-

Table II. Representative genes highly up-regulated by adenosine in dermal papilla cells.

Accession no.	Gene name	Abbreviation	Fold induction
NM_002009	Fibroblast growth factor 7	FGF-7	4.97±1.77
NM_002006	Fibroblast growth factor 2	FGF-2	3.13±0.85

[*]DP cells were treated with 100 μM adenosine for 6 h. Total-RNAs were isolated and used for preparation of Cy3- and Cy5-labeled probes.
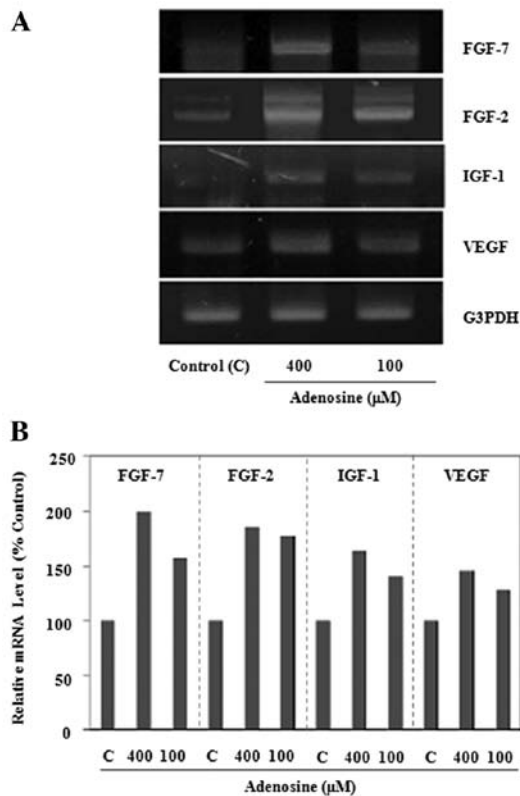

Figure 4. Effect of adenosine on the mRNA level of growth factors in cultured human hair dermal papilla cells based on semi-quantitative RT-PCR analysis. (A) DP cells were cultured with adenosine at the indicated concentrations for 18 h. Total-RNA was isolated from both control DP cells and cells treated with adenosine at the indicated concentrations after 18 h, reverse-transcribed, and a polymerase chain reaction was performed for fibroblast growth factor receptors 7 and 2 (FGF-7, FGF-2), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). (B) Quantification of RT-PCR products for each growth factor using a densitometer. The amount of RT-PCR product for each growth factor was corrected according to the quantity of G3PDH, and expressed as a percentage of a control.
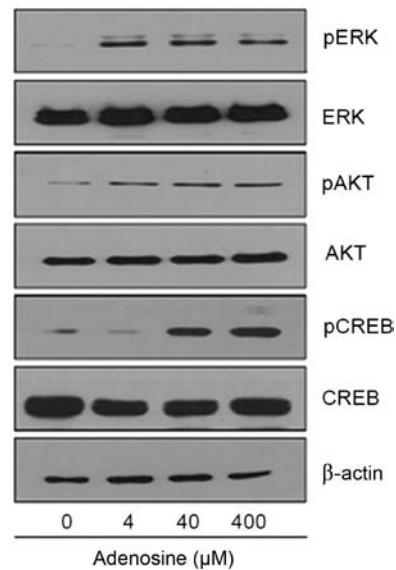

Figure 5. Effect of adenosine on phosphorylation of p42/44 ERK, CREB and AKT. DP cells were lysed in a Proprep solution after adenosine treatment at different concentrations for 30 min. The total protein content from cell lysates was analyzed using primary antibodies for total-p38 MAPK, phospho-p38 MAPK, total-p42/44 ERK, phospho-p42/44 ERK, total-AKT, phospho-AKT, total-CREB, phospho-CREB and β-actin.

peutic methods to stop hair loss and to enhance hair growth. Adenosine has been suggested as a potent regulator of hair growth. It stimulates VEGF production through activation of cell surface adenosine receptors, and this adenosine-mediated signal transduction pathway contributes to minoxidil-induced hair growth (9). Gene expression of FGF-7 was up-regulated by adenosine treatment in DP cells (10). Oura *et al* (17) demonstrated that adenosine significantly increased the anagen hair growth rate and the thickness of hair in a human clinical study. However, the adenosine hair growth stimulatory effect on hair follicle treatment and the underlying mechanism have not been fully elucidated.

In this study, we have demonstrated that adenosine significantly influences maintenance of the anagen phase of hair follicle activity, and stimulates hair follicle growth in an organ culture system. Adenosine also shows a stimulatory effect on the proliferation of human hair DP cells cultured *in vitro*. DP cells are known to be responsible for induction and maintenance of growth, and differentiation of epithelial cells in the hair bulb (18). The morphology of DP cells can be altered through the hair growth cycle, being maximal in volume in the anagen and least at the telogen phase. This is, in fact, a result of changes in the cell number and the amount of extracellular matrix within the cells (19). Thus, the proliferative potential of hair follicles and DP cells is thought to be an important parameter that regulates hair growth. In this regard, our results indicate that adenosine stimulates hair growth by inducing proliferation of hair follicular cells.

We performed DNA microarray analysis of DP cells and found that adenosine stimulation increases FGF-2 and FGF-7 expression by more than 2-fold. FGF-7 is known to induce and maintain anagen hair follicle growth. FGF-7 expression in the hair follicle dermal papilla was up-regulated during the anagen phase (20). Furthermore, addition of FGF-7 to a hair organ culture medium causes a decrease in the number of follicles in the catagen phase compared to the number of follicles in an

untreated medium (10). FGF-2 also has been reported to function in hair follicles. The FGF-1 and FGF-2 receptors (FGFR-1 and FGFR-2) were detected in DP cells, and FGF-2 enhances DPC-mediated outer root sheath cell proliferation (21). FGF-2 is essential for long term culture of dermal papilla cells and for the sphere formation that is a partial model of an intact DP, resulting in hair follicle induction (22,23). Therefore, our results suggest that adenosine triggers signal transduction cascades to mediate proliferation by regulation of the gene expression levels of FGF-7 and FGF-2 in cultured DP cells.

Our experimental results also show that adenosine promotes hair growth by up-regulating IGF-1 and VEGF gene expression. Several growth factors are reported to have important roles in hair growth. IGF-1, VEGF, keratinocyte growth factor (KGF), and HGF have stimulatory effects, whereas epidermal growth factor (EGF) and TGF-β have inhibitory effects on hair follicle growth (24). IGF-1 has important biological activities in various cell types, including hair follicles, such as promotion of growth and differentiation. In IGF-1 transgenic animals, there were many more hair follicles and elongated hair shafts, compared with non-transgenic littermates (25). VEGF also plays important roles in the angiogenesis that is associated with the hair growth cycle (26,27). VEGF mRNA expression in normal human hair follicles varies during the hair cycle. It is strongly expressed in the anagen phase and decreased in the catagen and telogen phases (28,29). Our experimental results, therefore, suggest that treatment of DP cells with adenosine is effective in maintaining high IGF-1 and VEGF expressions and the ability to induce hair growth in DP cells.

Since Wnt/β-catenin signaling has been proposed to function in hair follicle morphogenesis and differentiation (30,31), we investigated the effect of adenosine on the transcriptional activity of β-catenin in DP cells. Adenosine leads to an increase in the transcriptional activity of β-catenin. The activation of β-catenin in DP cells stimulates positive changes in hair follicles. Activated β-catenin is known to act in concert with LEF/TCF DNA-binding proteins to transactivate downstream target genes, involving the ERK pathway (32). Therefore, we investigated adenosine stimulation of DP cell growth via activation of protein kinase pathways. Adenosine leads to phosphorylation of the transcription factors ERK, CREB and AKT. These results suggest that adenosine effects a variety of cellular functions by triggering multiple intracellular signal transduction pathways. Taken together, our results support the hypothesis that adenosine increases hair growth related to expressions of the FGF-7 and FGF-2 genes via β-catenin and mitogenic signaling.

Acknowledgements

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