Adenosine Stimulates Fibroblast Growth Factor-7 Gene Expression *Via* Adenosine A2b Receptor Signaling in Dermal Papilla Cells

Masato Iino[1,4], Ritsuko Ehama[1,4], Yosuke Nakazawa[1], Tokuro Iwabuchi[1], Masashi Ogo[1], Masahiro Tajima[2] and Seiji Arase[3]

It has been previously reported that an adenosine receptor-mediated signal-transduction pathway in the dermal papilla cells (DPCs) of hair contributes to minoxidil-induced hair growth. In this study, we investigated this hypothesis further and have elucidated some underlying mechanisms. We performed DNA microarray analyses of DPCs and found that adenosine stimulation increases fibroblast growth factor-7 (FGF-7) gene expression levels by greater than 2-fold. Elevations of the extracellular FGF-7 protein levels were also observed. These upregulations of FGF-7 both at mRNA and protein levels were inhibited by A2b adenosine receptor-specific antagonist, alloxazine, but not by antagonists for other subtypes. In addition, the intracellular cAMP levels were raised by adenosine in a dose-dependent manner. Moreover, an increase of intracellular cAMP augmented the FGF-7 upregulation. Taken together, these results show that adenosine treatment of DPCs upregulates FGF-7 expression via the A2b adenosine receptor and that cAMP acts as one of the second messengers in this pathway. Furthermore, treatment with FGF-7 at concentrations of 10 ng/ml or greater significantly stimulated hair fiber elongation in human scalp hair follicle organ cultures. These data imply that adenosine might stimulate hair growth through FGF-7 upregulation in DPCs.

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INTRODUCTION

A series of closely coordinated epithelial–mesenchymal interactions in skin plays a central role not only in the organogenesis of the hair follicles, but also in the pathways that occur during hair follicle cycling (reviewed by Botchkarev and Kishimoto, 2003). The hair follicle cycle comprises repeated periods of hair growth and hair fiber formation (anagen), apoptosis-driven involution (catagen), and both hair regression and hair shedding (telogen-exogen) (reviewed by Stenn and Paus, 2001). The continuation and the transition of each phase results from the molecular control of epithelial–mesenchymal interactions by factors such as Wnt, transforming growth factor-β, bone morphogenetic proteins, notch, hedgehog, fibroblast growth factors (FGFs), epidermal growth factor, tumor necrosis factor, and the neurotrophin family. Several studies of hair follicle organ cultures have confirmed that insulin-like growth factor-1, hepatocyte growth factor, and macrophage-stimulating protein stimulate hair fiber growth via specific receptors on epithelial cells, insulin-like growth factor-1R, c-MET, and RON, respectively (Jindo *et al.*, 1994; Itami *et al.*, 1995; Rudman *et al.*, 1997; Lindner *et al.*, 2000; McElwee *et al.*, 2004). In addition, it has been shown that transforming growth factor-β inhibits hair growth (Inui *et al.*, 2002; Soma *et al.*, 2002). The stimulatory factors listed above are now considered to be powerful inducers of hair growth and transforming growth factor-β is believed to be the principal modulator of the transition from the anagen phase to the catagen phase.

Minoxidil has been reported to be a powerful inducer of hair growth because it causes significant hypertrichosis in humans (reviewed by Messenger and Rundegren, 2004). Lachgar *et al.* (1998) previously found that the production of vascular endothelial growth factor (VEGF) is enhanced by minoxidil in cultured dermal papilla cells (DPCs), and concluded that minoxidil promotes hair growth via this induction. Li *et al.* (2001) have proposed a mechanism for minoxidil-induced VEGF production by which an adenosine-mediated signaling pathway contributes to minoxidil-induced hair growth. Minoxidil sulfate is known to activate and open the sulfonylurea receptors of DPCs and cause the secretion of adenosine 5′-triphosphate. Adenosine 5′-triphosphate digestion by ecto-ATPase increases extracellular adenosine.

[1]*Shiseido Research Center, Yokohama, Japan;* [2]*R-Tech Ueno, Ltd., Chiyoda-ku, Tokyo, Japan and* [3]*Department of Dermatology, School of Medicine, The University of Tokushima, Tokushima, Japan*

[4]*These authors contributed equally to this work.*

Correspondence: Dr Masahiro Tajima, R-Tech Ueno, Ltd. 10F, Yamato Life Insurance Building, 1-1-7 Uchisaiwaicho, Chiyoda-ku, Tokyo 100-0011, Japan. E-mail: masahiro.tajima@rtueno.co.jp

Abbreviations: AdoR, adenosine receptor; ALX, alloxazine; CPX, 8-cyclopentyl-1,3-depropylxanthine; CSC, 8-(3-chlorostyryl) caffeine; DPC, dermal papilla cell; EC_{50}, median effective concentration; FGF, fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse-transcription PCR; VEGF, vascular endothelial growth factor

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Consequently, the upregulation of VEGF in DPCs is induced through adenosine receptor (AdoR) pathways. However, the mechanisms of hair growth governing the production of VEGF by minoxidil have not been clearly elucidated.

In this study, we have further elucidated AdoR signal transduction in DPCs. Adenosine is known to have various physiological functions, including the inhibition of lipolysis, vasodilation, and protection against ischemic damage, that are mediated via the up- or downregulation of cAMP, IP_3, and IP_3/diacylglycerol through the G protein coupling receptors of the four AdoR subtypes A1, A2a, A2b, and A3 (reviewed by Fredholm *et al.*, 2001). To further understand the molecular basis of hair growth mechanisms, we investigated the effects of adenosine on the expression of growth factors in cultured DPCs derived from human scalp hair follicles. DNA microarray analysis revealed the upregulation of several growth factors, including fibroblast growth factor 7 (FGF-7), following adenosine stimulation. We confirmed the increase in FGF-7 gene and protein expression using real-time reverse-transcription PCR (RT-PCR) and specific antibodies, respectively. We have also characterized the intracellular secondary signal messenger in this pathway and show that FGF-7 stimulates hair growth in organ cultures of human hair follicles.

RESULTS

The AdoR A2b receptor facilitates FGF-7 upregulation by adenosine in DPCs

We performed DNA microarray analyses (on a total of 12,814 genes) of DPCs that were incubated with or without 10 μM adenosine for 4 hours. Adenosine stimulation induced over 2-fold increases in VEGF gene expression in accordance with a previous report (Li *et al.*, 2001). In addition, FGF-7 gene expression level was found to be upregulated up to 2.28- and 2.57-fold. In contrast, the expression of other FGFs or paracrine growth factors such as FGF-1, FGF-2, FGF-9, FGF-12, insulin-like growth factor-1, and insulin-like growth factor-2 were not significantly affected by adenosine treatment.

FGF-7 is one of the most abundantly induced genes during the development of hair follicles (Werner *et al.*, 1994) and is considered as an important endogenous mediator of normal hair follicle growth, development, and differentiation (Danilenko *et al.*, 1995). To confirm the upregulation of FGF-7 gene expression by adenosine stimulation of DPCs, dose dependency was examined using quantitative real-time RT-PCR. The expression levels of FGF-7 were found to be significantly upregulated by concentrations of adenosine above 10 μM and to reach a peak induction level of approximately 3.8-fold following treatment with 300 μM adenosine (Figure 1). The median effective concentration of adenosine (EC_{50}) for FGF-7 gene induction was estimated from the three independent experiments to be 26.7$\pm$7.8 μM. FGF-7 protein levels in the medium of the cultured DPCs were assayed by enzyme immunoassay and were measured at 112.7$\pm$7.7 pg/ml following 50 μM adenosine stimulation for 18 hours (Figure 2). This was significantly higher than the control (80.9$\pm$5.1 pg/ml; Figure 2).

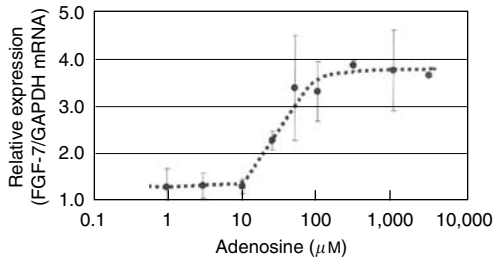

Figure 1. FGF-7 gene expression is upregulated following adenosine stimulation of DPCs. RNA was isolated from subconfluent cultures of DPCs (passage 3 or 4) that had been treated with adenosine for 2 hours and subjected to RT. Quantitative analysis of RNA was performed by real-time PCR in a LightCycler rapid thermal cycler system. The cycle numbers obtained for FGF-7 mRNA were normalized to GAPDH. The relative expression data were presented as the mean values$\pm$SD of the measurements that were normalized to the mean values obtained from control untreated cells ($n=3$). Ten μM to 3 mM of adenosine significantly stimulates FGF-7 gene expression in a dose-dependent manner ($P<0.05$ in Student's *t*-test). EC_{50} for this upregulation was estimated to be 27.1 μM. Similar results were obtained from two repeat experiments by using independently cultured cells, and the average EC_{50} in the three experiments was 26.7$\pm$7.8 μM.

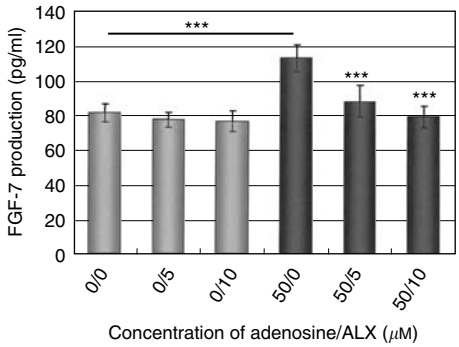

Figure 2. FGF-7 induction in DPCs by adenosine is suppressed by the A2b receptor antagonist ALX. Confluent DPCs were cultured on multi-well plates with or without adenosine and/or ALX for 18 hours. After centrifugation, FGF-7 levels in the supernatants were assayed by enzyme immunoassay. The results ($n=8$) presented are the mean values$\pm$SD. The *P*-values were determined by the Student's *t*-test from the differences between the test samples and the positive control cells that had been treated with 50 μM adenosine alone. ***$P<0.001$. An independent experiment revealed similar results ($n=8$); the base line was 57.8$\pm$4.3 pg/ml. Fifty μM of adenosine significantly induced a 1.29-fold increase ($P<0.001$). The addition of 5 μM ALX resulted in 71% inhibition ($P<0.01$) of the adenosine-induced FGF-7 production.

To determine the subtype of adenosine signaling receptor (AdoR) that mediates the upregulation of FGF-7, we employed receptor antagonists. The inhibitory effects on FGF-7 induction by adenosine were assayed in DPCs using specific antagonists for four types of AdoR. An AdoR A2b-specific antagonist alloxazine (ALX, Figure 3c) significantly suppressed the upregulation of FGF-7 following 25 μM adenosine stimulation. In contrast, neither 8-cyclopentyl-1,3-depropylxanthine (CPX, Figure 3a), 8-(3-chlorostyryl) caffeine (CSC, Figure 3b) nor 3-ethyl-5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS-1191, Figure 3d), which block the activity of

AdoR A1, A2a, and A3, respectively, has any effect upon FGF-7 upregulation. We contend that the suppression of adenosine-induced FGF-7 by a 500 nM dose of CPX is attributable to the nonspecific binding of this antagonist to AdoR A2b at concentrations above 50 nM (Linden *et al.*, 1999). The elevated FGF-7 protein levels in the medium of DPCs by 50 μM adenosine were also significantly reduced by cotreatment with 5 and 10 μM of ALX (Figure 2). These findings suggest that an AdoR A2b-mediated signal-transduction pathway in the DPCs contributes to FGF-7 upregulation by adenosine.

The AdoR A2b is present on hair follicles

To further examine the involvement of AdoR A2b signaling in dermal papilla, we performed immunohistochemical staining for this receptor on human hair follicles (Figure 4). AdoR A2b proteins were mainly detectable on both the dermal papilla and the outer root sheath, and were evident at lower levels on the dermal sheath and the hair matrix cells.

cAMP acts as one of the second messengers for the FGF-7 upregulatory signaling pathway through AdoR A2b in DPCs

It has been shown that AdoR A2b generally couples with the Gs protein or, in certain cell types, the Gq/11 protein, and that its second messenger in these instances is cAMP and IP_3/diacylglycerol, respectively (Pierce *et al.*, 1992; Gao *et al.*, 1999). To determine the identity of the second messenger that mediates FGF-7 upregulation by adenosine in DPCs, we analyzed the intracellular cAMP levels in these stimulated cells by enzyme immunoassay. We subsequently observed that the intracellular cAMP levels in DPCs were significantly increased following 5 minutes incubation with 25 μM adenosine (data not shown). Moreover, these intracellular cAMP levels increased in a dose-dependent manner over concentrations of 10 μM (Figure 5a). It was noteworthy that both this

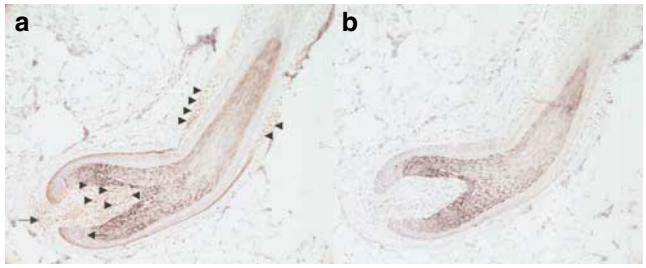

Figure 4. Immunohistochemical staining of AdoR 2b on a human scalp specimen. A human scalp specimen from a healthy 25-year-old male subject was fixed with 10% formalin in phosphate-buffered saline, pH 7.4 and embedded in paraffin. (**a**) Five μm sections were incubated with 20 μg/ml of rabbit polyclonal antibody against human AdoR A2b (AB1589P). (**b**) Substitution of the primary antibodies with normal rabbit IgG served as a negative control and the color reactions were developed using 3,3′-diaminobenzidine. Note that AdoR A2b was mainly detectable on the dermal papilla and the outer root sheath (indicated by arrowheads), and found to be present at low levels on the dermal sheath and the hair matrix cells (indicted by arrows).

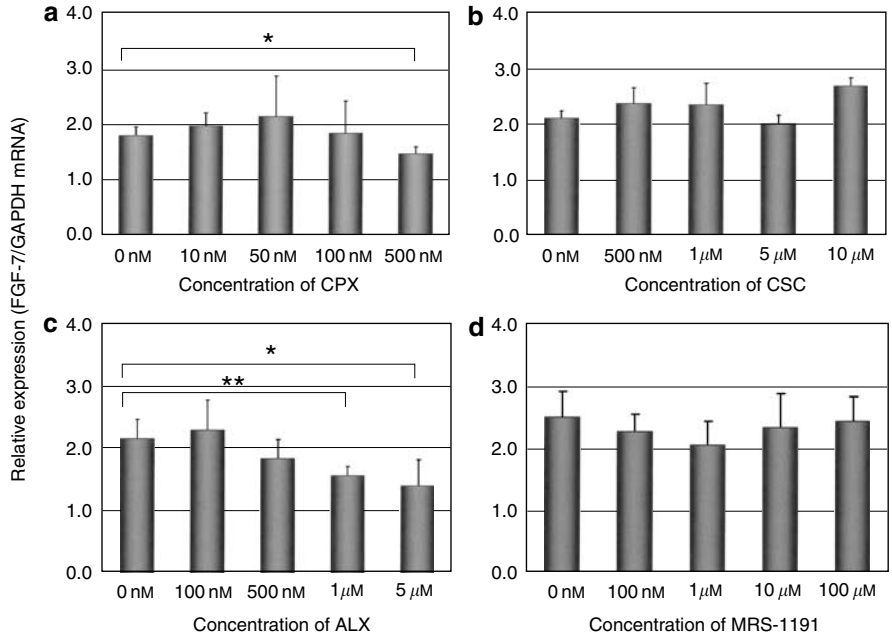

Figure 3. The effects of different AdoR antagonists upon adenosine-stimulated FGF-7 gene upregulation in DPCs. RNA was isolated from subconfluent cultures of DPCs (passage 3 or 4) that had been incubated with or without 25 μM adenosine and AdoR antagonists for 2 hours. Real-time RT-PCR was then performed. Cycle numbers obtained for FGF-7 mRNA were normalized to GAPDH. Relative expression data are presented as the mean values $\pm$SD ($n=4$), normalized to the controls without adenosine. It is noteworthy that the (**c**) AdoR A2b-specific antagonist ALX, (**a**, **b**, and **d**) but not the antagonists specific for AdoR A1 (CPX), A2a (CSC), or A3 (MRS-1191), suppresses the upregulation of FGF-7 gene expression. The *P*-values were determined by the Student's *t*-test from the differences between the test samples and the positive control cells that had been treated with 25 μM adenosine alone. $^*P<0.05$, $^{**}P<0.01$. These experiments were repeated and generated similar results; the addition of 5 μM ALX resulted in 72% inhibition ($P<0.05$) of the 25 μM of adenosine-induced FGF-7 gene expression (1.98$\pm$0.49-fold increase). In contrast, the addition of CPX, CSC, or MRS-1191 generated no meaningful inhibition.

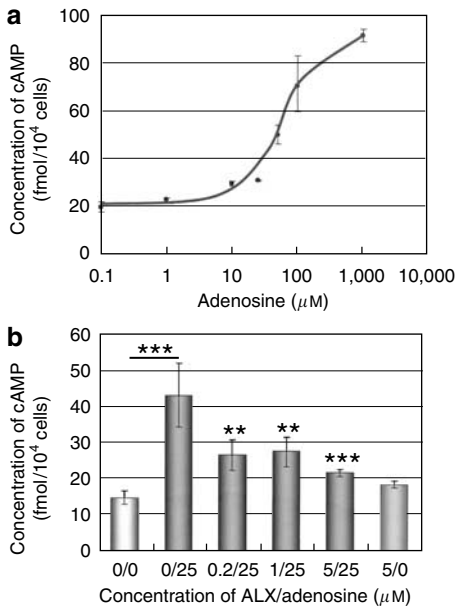

Figure 5. Upregulation of cAMP following adenosine stimulation of DPCs. DPCs (passage 4) were incubated with or without adenosine and AdoR antagonists for 20 minutes at 37°C. The intracellular cAMP concentration was then determined using an enzyme immunoassay kit. The results shown represent the mean values ± SD ($n = 6$). (**a**) Upregulation of intracellular cAMP levels following adenosine stimulation of DPCs. The addition of 10 μM adenosine and above significantly increased the cAMP levels ($P < 0.05$ by Student's *t*-test). EC_{50} for this upregulation was estimated to be 64.8 μM. Similar results were obtained from two repeat experiments and the average EC_{50} in the three experiments was 42.7 ± 17.8 μM. (**b**) The effects of the AdoR A2b-specific antagonist, ALX, upon intracellular cAMP upregulation by adenosine. ALX concentrations of 200 nM and above significantly suppressed the cAMP upregulation by adenosine. The *P*-values were determined by the Student's *t*-test from the differences between the test samples and the positive control cells that had been treated with adenosine alone. $^{**}P < 0.01$, $^{***}P < 0.001$. Two independent experiments revealed similar results; the base lines were 24.0 ± 0.9 and 30.1 ± 5.3 fmol/10^4cells. Twenty-five μM of adenosine significantly induced 2.90- and 3.55-fold increase ($P < 0.01$). The addition of 1 μM ALX resulted in 75 and 63% significant inhibition ($P < 0.05$ and $P < 0.01$, respectively) of the cAMP upregulation.

dose-dependent pattern and the estimated EC_{50} of 42.7 ± 17.8 μM from three independent experiments were similar to the values measured during the upregulation of FGF-7 gene expression in DPCs (Figure 1). Furthermore, the specific AdoR A2b antagonist ALX suppressed the induction of cAMP by 25 μM adenosine stimulation (Figure 5b), which was comparable with the suppression of FGF-7 gene upregulation by this inhibitor (Figure 3c). In contrast, the addition of specific antagonists for AdoR A1 (CPX), A2a (CSC), and A3 (MRS-1191) did not affect increased cAMP level (data not shown).

If the second messenger for the FGF-7 upregulation is cAMP, we reasoned that either cAMP analogs, or substances that are known to elevate intracellular cAMP levels, should also upregulate FGF-7 gene expression. We observed indeed that an adenylate cyclase activator, forskolin, significantly stimulates FGF-7 gene expression in a dose-dependent manner at a concentration range of 100 nM to 1 μM (Figure 6).

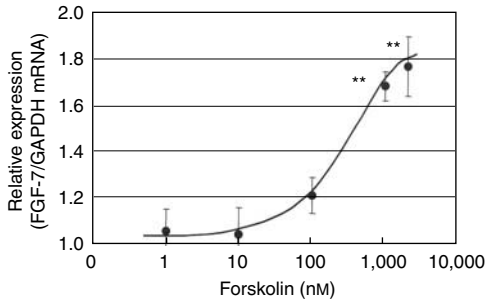

Figure 6. FGF-7 gene expression is upregulated by forskolin stimulation of DPCs. RNA was isolated from subconfluent cultures of DPCs (passage 3) treated with forskolin for 2 hours, and was subjected to real-time RT-PCR. The cycle numbers obtained for FGF-7 mRNA were normalized to GAPDH. The relative expression data are presented as the mean values ± SD ($n = 4$), normalized to untreated control. Forskolin concentrations of 1 μM and higher significantly stimulate FGF-7 gene expression in a dose-dependent manner ($^{**}P < 0.01$ by Student's *t*-test).

A cAMP analog, dibutiryl-cAMP, also produced these effects at doses from 10 μM to 1 mM (data not shown). Taken together, these results indicate that cAMP is generated via the Gs protein coupling receptor, AdoR A2b, and acts as one of the second messengers that mediate FGF-7 upregulation in DPCs.

The effects of FGF-7 upon cultured human hair follicles

To further understand the contribution of FGF-7 during hair growth, the effects of the human FGF-7 protein were assayed using human anagen hair organ cultures. The addition of 10 ng/ml (Days 6 and 9), 30 ng/ml (Days 6 and 9), and 100 ng/ml (Days 3, 4, 5, 6, and 9) of FGF-7 into the culture medium resulted in significant hair elongation when compared with the control cultures over 9 days (Figure 7). Moreover, the hair follicles treated with FGF-7 maintained anagen morphology during this 9-day period. These results indicate that FGF-7 may stimulate hair growth by inducing the proliferation and differentiation of human hair follicular cells.

DISCUSSION

It has been reported that an adenosine-mediated signal-transduction pathway, which is in part mediated by intracellular Ca^{2+}, contributes to minoxidil-induced hair growth. This evidence is derived from studies showing minoxidil-induced VEGF production in cultured DPCs (Li *et al.*, 2001). We now show from our current experiments that the gene expression level of FGF-7 is upregulated in cultured DPCs by a 10 μM concentration of adenosine. FGF-7, a glycoprotein secreted from mesenchymal cells, has a target specificity that is restricted to the mitotic activity of epithelial cells. In addition, as a paracrine growth factor, FGF-7 functions during the morphogenesis of epithelia, the re-epithelialization of wounds, and during hair development (reviewed by Danilenko, 1999). It has been shown to directly affect the development of hair follicles (Werner *et al.*, 1994) and is known as its antagonistic interplay with the differentiation factor, bone morphogenetic protein 2/4 (Kulessa *et al.*, 2000; Kobielak *et al.*, 2003). Rosenquist and Martin (1996) previously localized FGF-7 mRNA in the dermal papilla of the hair follicles during anagen, whereas FGF-7

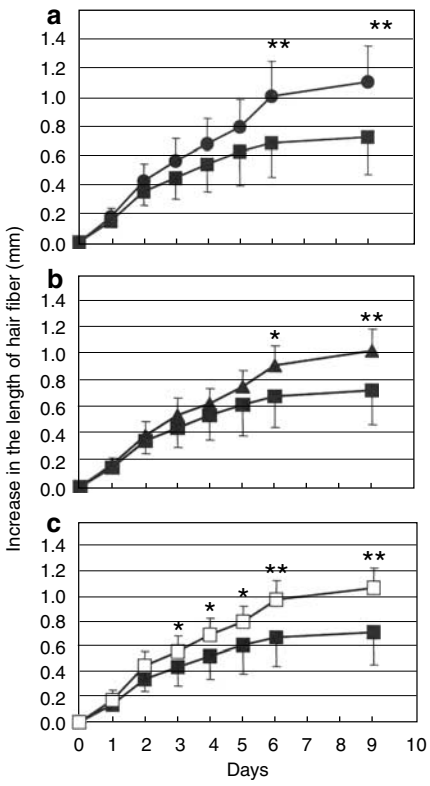

Figure 7. The effects of FGF-7 upon hair elongation in follicle organ cultures from human scalp. Anagen hair follicles were isolated from human scalp and were incubated with recombinant human FGF-7 at concentrations of (**a**, **b**, and **c**) 0 ng/ml (containing vehicle 0.01% DMSO; ■), (**a**) 10 ng/ml (●), (**b**) 30 ng/ml (▲), or (**c**) 100 ng/ml (□) ($n=13$, in each concentration). Increases in the length of hair fibers were measured sequentially over 9 days with an inverted microscope that had been fitted with an eyepiece measuring graticule. The results represent the mean ± SD of the increases in hair fiber lengths, compared with the baseline data. Statistical analyses of these data were carried out using the Student's *t*-test. $*P<0.05$, $**P<0.01$. An independent experiment ($n=14$–18) revealed similar results; addition of 100 ng/ml of FGF-7 significantly stimulated the hair fiber growth ($P<0.05$) at Days 5, 8, and 11.

mRNA levels were below the limits of detection elsewhere in the skin. Further, expression was downregulated by the late anagen VI stage.

Exogenously administered FGF-7 was previously shown to stimulate hair growth in nu/nu athymic nude mice by stimulating follicular proliferation and inducing normalization of the nu/nu follicular keratin differentiation defect (Danilenko *et al.*, 1995). Sredni *et al.* (2004) also reported that the synthetic immunomodulator AS101, ammonium trichloro (dioxoethylene-*o*,*o*′) tellurate, stimulates hair growth and its stimulation depends entirely on FGF-7. AS101 possesses the dual ability to both induce anagen and retard spontaneous catagen in mice. Anagen induced by AS101 is thought to be mediated by FGF-7, as it is abrogated both in nude mice cotreated with AS101 plus neutralizing anti FGF-7 antibodies and in AS101-treated transgenic mice expressing a dominant-negative FGF-7 receptor transgene in basal keratinocytes.

FGF-7 has also been shown to inhibit the transition to catagen shapes in hair follicle organ cultures (Philpott, 2000). The addition of 10 ng/ml FGF-7 to hair organ culture medium for 7 days causes a decrease (58%) in the number of follicles in catagen when compared with the follicles (73%) in untreated medium. In this study, the stimulation of hair fiber elongation was observed with daily addition of 10, 30, and 100 ng/ml FGF-7 to the culture medium. Taken together, these data suggest that FGF-7 induces and maintains hair growth at anagen.

There are no earlier reports regarding the effect of adenosine on hair follicle organ culture. We observed in hair organ cultures over a period of 5 days that 1 μM (21%, $n=14$, $P=0.0066$) and 100 μM (14%, $n=14$, $P=0.0183$) of adenosine, but not 10 nM adenosine (43%, $n=14$, $P=0.3661$), produced significant decreases in the ratio of hair follicles of catagen-like shape, compared with control follicles (64%, $n=14$) (Y Nakazawa, unpublished data). The anagen maintaining effects of adenosine may be due to the effect of FGF-7 induced by adenosine on DPCs.

We also investigated the intracellular signal-transduction mechanisms underlying adenosine stimulation of cultured DPCs. Adenosine interacts with the four G protein-coupled receptors, A1, A2a, A2b, and A3 (Fredholm *et al.*, 2001). The common effector of the response to the activation of these receptors is adenylate cyclase, which is negatively regulated by A1 and A3 receptors and positively regulated by A2 receptors. AdoR A2b is characterized as a ''low-affinity'' receptor for adenosine. The EC_{50} value for FGF-7 upregulation by adenosine in DPCs was calculated to be 26.7 μM, suggesting that the signaling receptor for this process was AdoR A2b because it requires high concentrations of adenosine (EC_{50}, 5–10 μM) to facilitate the pathway (Hide *et al.*, 1992). In contrast, the other three AdoR subtypes are generally activated at much lower levels (~0.1 μM). Our competition experiments with AdoR antagonists for both transcription and translation of FGF-7 provided evidence of the contribution of AdoR A2b to the upregulation of this gene by adenosine stimulation. Furthermore, immunochemical staining of AdoR A2b in the dermal papilla of hair supports the contention that this receptor participates in FGF-7 gene expression in these cells *in vivo*.

The downstream signaling pathways of AdoR A2b in DPCs are still somewhat unclear. The receptor is shown to be coupled with the Gs and Gq proteins (Auchampach *et al.*, 1997; Linden *et al.*, 1999) and thus contribute to the upregulation of cAMP and IP_3/diacylglycerol, respectively. Significantly, the upregulation of cAMP through AdoR A2b was observed following adenosine stimulation in this study. Moreover, both forskolin and dibutiryl-cAMP caused the upregulation of FGF-7 gene expression. These data demonstrate that the intracellular cAMP signal pathway, at least in part, contributes to the upregulation of FGF-7 gene by adenosine stimulation. cAMP is a second messenger that is produced in cells in response to growth factors, neurotransmitters, and hormones. It is known that the activation of AdoR A2b stimulates a cAMP-dependent protein kinase pathway and thus promotes the phosphorylation of various proteins,

including the transcription factor cAMP response element-binding protein (reviewed by Mayr and Montminy, 2001). A number of cAMP-dependent signaling pathways have been characterized and shown to be mediated primarily by cAMP response element-binding protein/activating transcription factor family members, which are basic leucine zipper transcription factors. The cAMP response element-binding protein/activating transcription factor family of transcription factors binds to the cAMP responsive element consensus sequence TGACGTCA. Brauchle *et al.* (1994) have reported that FGF-7 mRNA and protein expression is at low levels in quiescent fibroblasts but is strongly induced upon serum stimulation, suggesting the participation of at least two different intracellular pathways involving protein kinase C or cAMP-dependent kinase. Zhou and Finch (1999) identified a cAMP responsive element-like regulatory element, TGAGGTCAG between −39 and −46 bp relative to the transcription start site of the human FGF-7 basal promoter region, which directs gene transcription in a fibroblast-specific manner and binds to the inducible activating transcription factors. Hence, it would be reasonable to predict that cAMP response element-binding protein/activating transcription factor family members are involved in the upregulation of FGF-7 in DPCs by adenosine.

Recently, we performed a randomized clinical test for the evaluation of adenosine as a hair growth promoter. Of the 102 Japanese men with androgenetic alopecia that we tested, a topical adenosine lotion was significantly superior to a control lotion in promoting an increase in thick hair (Watanabe *et al.*, paper submitted). Adenosine is known to have various physiological functions such as vasodilatation, anti-inflammation, angiogenesis, and tissue protection in vascular or neuronal cells; however, little is known about function in hair follicular cells (Fredholm *et al.*, 2001). In addition to the possibilities of the involvement of these action by adenosine in hair growth stimulation, we concluded from our presented data that adenosine might stimulate hair growth by the upregulation of FGF-7 in DPCs; however, further investigations will be needed to confirm the roles of adenosine in hair growth *in vivo*.

MATERIALS AND METHODS

Reagents, tissues, and cells

Reagents containing the AdoR antagonists, CPX (Klotz *et al.*, 1998), CSC (Jacobson *et al.*, 1993), ALX (Brackett and Daly, 1994), 3-ethyl-5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS-1191, Muller, 2001), dibuthyl-cAMP, forskolin, and recombinant human FGF-7 protein were purchased from Sigma™ (Sigma-Aldrich Japan KK, Tokyo, Japan). Adenosine was purchased from Yamasa Corporation (Chiba, Japan). Scalp skin samples were provided by volunteers with informed consent. The subcutaneous fat portion where hair follicles reside was collected, and anagen hair follicles were isolated. DPCs were separated from the hair follicles and cultured in DMEM (Gibco-BRL, Invitrogen Japan KK, Tokyo, Japan) supplemented with 10% fetal bovine serum at 37°C in the humidified atmosphere containing 5% CO_2. DPCs were subcultured after they had grown out from the papilla and achieved subconfluence. This study was approved by the Shiseido Ethics Committee to ensure subject protection and adhered to the Declaration of Helsinki Principles.

DNA microarrays

Confluent DPC cultures were incubated in DMEM with or without 10 μM adenosine for 4 hours. Total RNA was extracted with ISOGEN (Nippon Gene Co. Ltd, Tokyo, Japan) according to the manufacturer's instructions. Five μg of total RNA was then amplified by *in vitro* transcription (Luo *et al.*, 1999). The amplified RNA (6 μg) was reverse transcribed using random hexamers and aminoallyl-dUTP. The synthesized complementary DNA was then labeled with either *N*-hydroxysuccinimide ester Cy3 or Cy5 (Amersham Biosciences UK Ltd, Buckinghamshire, UK, Hughes *et al.*, 2001), applied to a complementary DNA microarray (Human 1, Agilent Technologies Inc., Palo alto, CA), and hybridized at 65°C for 17 hours. After washing, the microarray was scanned on a ScanArray™ 5000 and processed using QuantArray™ software (Packard Instrument Co., Meriden, CT). The intensity of each spot was calibrated by subtraction of the background signal. Global normalization methods were used to identify the differentially expressed genes from each microarray experiment. A gene was considered to be upregulated if the difference between the two intensities was greater than the background.

Real-time quantitative PCR

Subconfluent DPCs were cultured on multi-well plates with or without adenosine and an AdoR antagonist. mRNA was extracted with the MagNA Pure™ mRNA extraction kit and the MagNA Pure™ instrument (Roche Diagnostics Ltd, Tokyo, Japan) according to the manufacturer's instructions (mRNA 1 cells). The mRNA preparation was reverse-transcribed with SuperScript™II (Invitrogen Japan KK). Real-time quantitative PCR was performed on a LightCycler™ rapid thermal cycler system using LightCycler™ FastStart DNA master SYBR™ green I kit (Roche Diagnostics Ltd) according to the manufacturer's instructions. The FGF-7-specific primers used were as follows: 5′-CATGAACACCCGGAGCACTAC-3′ (forward, NM_002009: 419–439), 5′-CACTGTGTTCGACAGAAGAGTCTTC-3′ (reverse, NM_002009: 669–646). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a reference gene. To confirm the amplification specificity, PCR products were subjected to melting curve analysis. The expression levels of FGF-7 were analyzed quantitatively with the LightCycler™ analysis software (Morrison *et al.*, 1998). Fluorescence data were acquired at the end of each extension cycle and the background fluorescence was removed by setting a noise band. The crossing points, defined as the cycle number at which all samples were exponentially amplifying, were recalculated as relative log concentrations of RNA. The cycle numbers with respect to FGF-7 mRNA were normalized to GAPDH mRNA from the same extract. The mean value±SD of the GAPDH normalized FGF-7 expression was obtained from three or four samples in each condition. The results are presented as the relative ratio to the control with the absence of adenosine.

Determination of FGF-7 protein levels in cultured medium

The FGF-7 protein levels were measured by a commercial enzyme immunoassay kit (R&D Systems Inc., Minneapolis, MN), following the procedures provided by the supplier. Confluent DPC cultures on

multi-well plates were treated with or without adenosine and ALX, for 18 hours. After harvesting and centrifugation of the cells, the supernatants were assayed immediately. The results are presented as the mean values ± SD of eight samples in each condition.

Immunohistochemical staining

A human scalp specimen of a 25-year-old healthy male subject was fixed with 10% formalin in phosphate-buffered saline, pH 7.4 and embedded in paraffin. Sections (5 μm in thickness) were autoclaved in 10 mM citrate buffer (pH 6.0), and treated with 0.3% hydrogen peroxide, and then incubated with 20 μg/ml of rabbit polyclonal antibody against human AdoR 2b (AB1589P, Chemicon, Temecula, CA) in phosphate-buffered saline containing 12% BSA or normal rabbit IgG (negative control). Biotin-conjugated goat anti-rabbit IgG (Nichirei Corp., Tokyo, Japan) was used as the secondary antibody. AdoR A2b was visualized with VECTASTAIN™ Elite ABC Reagent (Vector Laboratories, Burlingame, CA) and a 3,3′-diamino-benzidine Substrate Kit (Vector Laboratories).

cAMP assay

The DPCs were inoculated at a concentration of 3×10^4 cells per well in standard 24-well plates and maintained in DMEM containing 10% fetal bovine serum for 3–4 days until they reached subconfluence. The cells were then serum starved for another 24 hours. Subsequently, the cells were incubated with or without adenosine and an AdoR antagonist for 20 minutes at 37°C. The intracellular cAMP concentrations were determined by a cAMP enzyme immunoassay kit (Biotrak™ cAMP EIA system, Amersham Biosciences UK Ltd) according to the manufacturer's protocol. The results are presented as the mean values ± SD of six samples in each condition.

Human hair follicle organ cultures

Human hair follicles were isolated and cultured using the method described by Philpott *et al.* (1990). Hair follicles isolated as described above were placed in individual wells of a 24-well multi-well plate, each maintained in 1 ml of Williams E medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml Fungizone (basal medium) (Gibco-BRL, Invitrogen Japan KK), supplemented with combinations of 10 ng/ml sodium selenite, 10 μg/ml transferrin, 10 ng/ml hydrocortisone, and 10 μg/ml insulin (plus medium) at 37°C in 5% CO_2/95% air. Twenty-four hours later, the medium was exchanged to basal Williams E medium with or without human recombinant FGF-7 (10, 30, or 100 ng/ml). The same medium was replaced every day for a further 8 days. The lengths of the hair fibers were measured over this 9-day period using a inverted microscope (Olympus IX70, Olympus Co., Tokyo, Japan) with an eyepiece measuring graticle at × 40 magnification.

Statistics

The differences in the data sets were analyzed by using the Student's *t*-test. *P*-values of <0.05 were considered to be statistically significant. The EC_{50} for FGF-7 upregulation and cAMP induction by adenosine were estimated using SAS (SAS institute Inc., Cary, NC) statistical software.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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