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Effect of topical application of raspberry ketone on dermal production of insulin-like growth factor-I in mice and on hair growth and skin elasticity in humans

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Abstract

Sensory neurons release calcitonin gene-related peptide (CGRP) on activation. We recently reported that topical application of capsaicin increases facial skin elasticity and promotes hair growth by increasing dermal insulin-like growth factor-I (IGF-I) production through activation of sensory neurons in mice and humans. Raspberry ketone (RK), a major aromatic compound contained in red raspberries (*Rubus idaeus*), has a structure similar to that of capsaicin. Thus, it is possible that RK activates sensory neurons, thereby increasing skin elasticity and promoting hair growth by increasing dermal IGF-I production. In the present study, we examined this possibility in mice and humans. RK, at concentrations higher than 1 μM, significantly increased CGRP release from dorsal root ganglion neurons (DRG) isolated from wild-type (WT) mice and this increase was completely reversed by capsazepine, an inhibitor of vanilloid receptor-1 activation. Topical application of 0.01% RK increased dermal IGF-I levels at 30 min after application in WT mice, but not in CGRP-knockout mice. Topical application of 0.01% RK increased immunohistochemical expression of IGF-I at dermal papillae in hair follicles and promoted hair re-growth in WT mice at 4 weeks after the application. When applied topically to the scalp and facial skin, 0.01% RK promoted hair growth in 50.0% of humans with alopecia ($n = 10$) at 5 months after application and increased cheek skin elasticity at 2 weeks after application in 5 females ($p < 0.04$). These observations strongly suggest that RK might increase dermal IGF-I production through sensory neuron activation, thereby promoting hair growth and increasing skin elasticity.

Keywords: Raspberry ketone; Sensory neurons; Calcitonin gene-related peptide; Insulin-like growth factor-I; Hair growth; Skin elasticity

1. Introduction

Insulin-like growth factor-I (IGF-I) is a basic peptide of 70 amino acids with a rather ubiquitous distribution in various tissues and cells, mediating the growth-promoting actions of growth hormone (GH) and playing an important role in postnatal and adolescent growth [1]. Furthermore, IGF-I has been shown to have various important biological activities such as promoting differentiation of various cell types, potent anti-apoptotic activity, and an anabolic effect [2].

Human skin has been shown to express genes for GH, GH-receptor and IGF-I [1,3], suggesting that the

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GH–IGF-I axis might have important functions in maintaining the integrity of the skin.

IGF-I is critically involved in promoting hair growth by regulating cellular proliferation and migration during the development of hair follicles [4]. Transgenic mice overexpressing IGF-I in the skin show earlier hair follicle development than controls [5]. Consistent with these observations is the fact that patients with Laron syndrome (primary IGF-I deficiency) have sparse hair growth and frontal recessions [6]. IGF-I has been shown to be produced by dermal papilla cells in hair follicles [7]. Since IGF-I receptor mRNA was detected in keratinocytes [8], it is possible that IGF-I produced by dermal papilla cells might act on keratinocytes, thereby promoting hair growth by stimulating the proliferation of these keratinocytes in hair follicles.

Since IGF-I has been shown to play an important role in the maintenance of normal skin morphology [9] and it increases collagen synthesis by fibroblasts *in vitro* [10], detrimental skin morphological changes observed in patients with Laron syndrome might be attributable to reduced IGF-I production.

Capsaicin-sensitive sensory neurons are nociceptive neurons and can be found in many tissues within the lining epithelia and around blood vessels [11]. These sensory neurons release calcitonin gene-related peptide (CGRP) upon activation of vanilloid receptor-1 (VR-1) by a wide variety of noxious physical and chemical stimuli [12], thereby showing sensory-efferent functions. CGRP, a 37-amino acid neuropeptide, is produced in dorsal root ganglion neurons (DRG) by tissue-specific alternative processing of the calcitonin gene [13]. We previously reported that sensory neuron activation increases IGF-I production in mice [14]. Furthermore, we recently demonstrated that topical application of capsaicin increases dermal levels of IGF-I, thereby increasing skin elasticity in humans [15]. In addition, sensory neuron activation leading to increased IGF-I production promotes hair growth in mice and humans [16].

Raspberry ketone (RK), a major aromatic compound contained in red raspberries (*Rubus idaeus*), has a structure similar to that of capsaicin [17] (Fig. 1). Thus, it is possible that RK increases dermal IGF-I production through sensory neuron activation, thereby promoting hair growth and increasing skin elasticity.

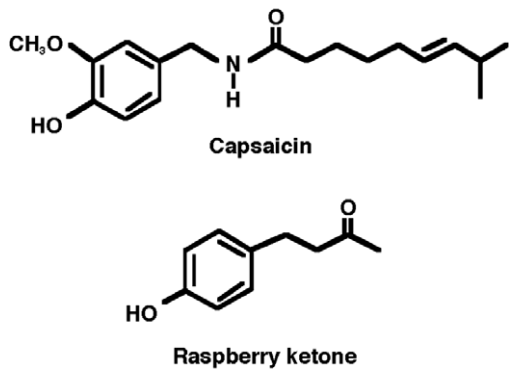

Fig. 1. Structural formulas of capsaicin and raspberry ketone.

In the present study, we attempted to determine whether topical application of RK increases dermal IGF-I levels through sensory neuron activation, thereby promoting hair re-growth in mice. We also examined the effect of topical application of RK to the scalp and facial skin on hair growth in humans with alopecia and on cheek skin elasticity in healthy female volunteers.

2. Methods

2.1. Reagents

RK with 99.9% purity is kindly provided by Kanebo Cosmetics Inc. (Odawara, Japan). Capsazepine (CPZ), an inhibitor of vanilloid receptor-1 activation [18], was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

2.2. Animals

Male C57BL/6 mice (6–8 weeks old; Nihon SLC, Hamamatsu, Japan) were used in this study. The care and handling of the animals were in accordance with the National Institute of Health Guidelines. All experimental procedures described below were approved by Nagoya City University Animal Care Committee.

2.3. Generation of αCGRP-knockout mice

The generation of αCGRP-knockout mice was described previously [19]. The mouse CT/αCGRP genomic DNA was cloned from a BALB/c mouse genomic library in EMBL3 using synthetic oligonucleotide probes derived from the mouse CT/αCGRP cDNA sequence. A 7.0-kb fragment containing exons 3–5 of the mouse CT/αCGRP gene was subcloned into pBluescript (Stratagene). A targeting vector was constructed by replacing the 1.6-kb XbaI–XbaI fragment encompassing exon 5, which is specific for αCGRP, with the neomycin resistance gene and flanking the thymidine kinase gene. This plasmid was linearized with NotI and introduced into 129/Sv-derived SM-1 ES cells by electroporation, after which the cells were selected in medium containing G418 (300 μg/mL) and ganciclovir (2 μmol/L). Homologous recombinants were identified by PCR and Southern blot analysis. Targeted ES cell clones were injected into C57BL/6 mouse blastocysts to generate chimeric mice. Male chimeras were then crossbred with C57BL/6 females and germline transmission was achieved. Littermates obtained by breeding heterozygotes with the genetic background of the 129/

SvXC57BL/6 hybrid were used for phenotypic analysis. Only males were used in this study.

2.4. Genotype determination of CGRP-knockout pups

Genomic DNA was extracted from tails of mice as previously described [19] and was used for PCR analysis. PCR was performed using the external primers of the replaced gene fragment. The wild-type allele and the mutant allele gave different band sizes. Primer sequences and PCR conditions have been described [19].

2.4.1. In vitro experiments

2.4.1.1. Isolation and culture of dorsal root ganglion neurons (DRG) and measurement of CGRP release. DRG isolated from the lumbar, cervical, and thoracic region were dissected from rats as described previously [20]. After 5 days in culture, the medium was aspirated gently and washed with serum-free Ham's F-12 medium. Cells were incubated with RK (1, 10, and 100 μM) for 30 min in Ham's F-12 medium containing 1% SCS without NGF. After incubation, supernatants were sampled and stored −20 °C for CGRP measurement. To determine whether RK increases CGRP release from DRG via vanilloid receptor-1 activation, we examined the effect of CPZ, an inhibitor of vanilloid receptor-1 activation [18], on RK-induced CGRP release from DRG. CGRP levels were determined using a commercial rat CGRP enzyme immunometric assay kit (SPI-Bio, Massy, France).

2.4.2. In vivo experiments

2.4.2.1. Protocol 1: Effect of single topical application of RK solution on dermal IGF-I levels in wild-type (WT) or CGRP-knockout mice

2.4.2.1.1. Topical application of RK solution. Mice were anesthetized with i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). RK was dissolved in 10% Tween 20/10% ethanol (10%) and diluted with normal saline. RK solution (100 μL) was applied to the shaved back of mice (4 cm^2) as described previously [15]. The skin was removed 30 min after topical application of RK solution, and then immediately immersed into liquid nitrogen.

2.4.2.1.2. Determination of dermal IGF-I level. Dermal levels of IGF-I were determined in animals by modification of the methods as described previously [21]. The skin was minced and homogenized in a polytron type homogenizer (two times of 15 s) using 2 mL of 1 N acetic acid according to the manufacturer's instruction. The homogenates were then centrifuged at 3000*g* for 10 min. The supernatants were kept in a deep freezer at −80 °C. The concentration of IGF-I was assayed by using a specific enzyme immunoassay kit (Diagnostic Systems Laboratories Inc., Webster, TX).

2.4.2.1.3. Measurement of tissue concentration of RK 30 min after topical application of RK solution. One hundred mg of treated skin was cut into small size and homogenized in a polytron type homogenizer (two times of 15 s). Then 50% alcohol solution was gradually added until getting the total preparation weight of 1000 mg. After centrifugation at 10,000*g* for 3 min, the supernatant was treated again with Amicon Ultra-4.5k (Millipore, Billerica, MA) at 4000*g* for 20 min at 4 °C to get optimized sample for the liquid chromatography–mass spectrometry (LC–MS/MS) analysis.

The high performance liquid chromatography (HPLC) system consisted of a binary pump, degasser, and auto-sampler Agilent Technologies Model 1200 (Minnesota, USA). A 3200Q-Trap mass spectrometer with Ion Source Turbo Spray from Applied Biosystems MSD Sciex (Toronto, Canada) was used. Nitrogen produced by a high-purity nitrogen generator (Air-Tech Inc., Yokohama, Japan) was used as curtain, nebulizer, collision, and lamp gases. Unit mass resolution was set in both mass-resolving quadrupole Q1 and Q3. Aliquots (1.0 μL) of standards or sample extracts were separated by HPLC on a column Inertsil ODS-3 (15 × 2.1 mm i.d., particle size 5 μm; GL Sciences, Tokyo, Japan). The mobile phase was aqueous mixed with acetonitrile 30% at a flow rate of 200 μL/min. The ion source was operated at 600 °C in the negative ion mode. Multiple reaction monitoring (MRM) mode was used and the collision energy was −22 V. The monitoring ions were as follows: the precursor ion was 163.003 $[M-H]^-$ and its diagnostic product ion was 57.108 $[M-HOC_6H_4CH_2]^-$. Data were collected using Analyst 1.4.1 software package (MDS SCIEX).

Stock solutions of 1.0 mg/mL in acetonitrile were prepared for RK. Working standard solutions at various concentrations were prepared by dilution with acetonitrile to obtain solutions with varying concentrations.

2.4.2.2. Protocol 2: Effect of topical application of RK solution once a day for 4 weeks on dermal IGF-I levels, immunohistochemical expression of IGF-I at dermal papillae in hair follicles, and hair growth in WT mice

2.4.2.2.1. Depilation treatment and topical application of RK solution. Mice were anesthetized with i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The dorsal areas (2 cm in width and 4 cm in length) of the mice were clipped with clippers. One hundred μL of 0.01% RK solution was applied to these areas once a day for 4 weeks.

2.4.2.2.2. Immunohistochemical staining of IGF-I. The peroxide–antiperoxide technique was used for immunohistochemical staining of various tissues with anti-IGF-I antibody [22]. Mice were perfused with 4% paraformaldehyde in phosphate buffer. Tissue blocks were immersed in the same perfusate at 4°C overnight and embedded in the paraffin wax by the ordinary manner

after several washings with phosphate buffer. Sections (6–8 μm thick) were mounted on glass slides. Samples were immersed in absolute acetone at −20 °C for 5 min, rinsed in PBS five times for 5 min each, and then incubated for 20 min with 10% porcine serum in PBS at room temperature. They were incubated for 1 h at 37 °C with rabbit anti-IGF-I polyclonal antibody at 1:100 dilution. After five rinses in PBS, the sections were treated with HRP-conjugated anti-rabbit (MBL Co. Nagoya, Japan) at 1:2000 dilution for 1 h at 37 °C. Reaction products were developed by immersing the sections in 3′3-diaminobenzidine tetrahydrochloride solution containing 0.03% hydrogen peroxide. The control for immunostaining was performed by non-immune rabbit serum, as first step in place of primary antiserum, and omission of the first step or use of the first antiserum preabsorbed with an excess of the homologous antigen. Samples were mounted with Entellan onto glass slides, examined, and photographed under light microscope.

2.4.3. Human experiments

2.4.3.1. Topical application of 0.01% RK to the scalp in human volunteers with alopecia. The protocol was approved by the Ethic Study Board of the Sugikami Hospital (Kumamoto Prefecture, Japan) where the study was performed. Informed consent was obtained from 10 volunteers (7 males and 3 females, aged 12–45 years). Among the volunteers with alopecia, 6 males suffered from androgenetic alopecia (AGA) and 4 from alopecia areata (AA) (1 male and 3 females). Topical application of 0.01% RK to the scalp was done once a day at night between 10 PM and 11 PM for 5 months. One of the investigators (Noriko Narimatsu) evaluated clinical efficacy by comparing the clinical condition at 5 months after the topical application of 0.01% RK to the scalp with the baseline photographs.

2.4.3.2. Topical application of 0.01% RK in healthy female human volunteers. The protocol was approved by the Ethic study Board of the Sugikami Hospital (Kumamoto Prefecture, Japan) where the study was done. Informed consent was obtained from 10 healthy female human volunteers (aged 36.8 ± 1.8 years). Topical application of 0.01% RK or vehicle containing Tween 20/10% ethanol (10%) and saline was done once a day (at night between 10 PM and 11 PM after washing their faces) for 14 days. We randomly assigned the 10 healthy female human volunteers to two groups: 5 received 0.01% RK and the remaining 5 received vehicle.

2.4.3.3. Assessment of skin elasticity after topical application of RK in human subjects. Before and after topical application of 0.01% RK or vehicle to 10 healthy female human volunteers for 14 days, we measured cheek elasticity by Cutometer MPA580 (Integral Co., Tokyo, Japan) as described previously [23]. In this system, skin elasticity is defined as A (maximal deformation)–B (basal level after removal of vacuum).

2.4.3.4. Statistical analysis. Data are expressed as the mean ± SD. The results were compared using an ANOVA followed by Scheffé's post hoc test. To determine the difference between values of skin elasticity obtained in the same subject before and after topical application of 0.01% RK, the statistical significance of the data was calculated by using the paired *t* test. A level of $p \leqslant 0.05$ was considered statistically significant.

3. Results

3.1. Effects of RK and/or capsazepine, an inhibitor of vanilloid receptor-1 (VR-1) activation, on CGRP release from DRG isolated from WT mice in vitro

To determine whether RK increases CGRP release from sensory neurons, we examined the effect of RK on CGRP release from DRG isolated from WT mice. RK, at concentrations higher than 1 μM, enhanced CGRP release from DRG (Fig. 2). Pretreatment with CPZ, an inhibitor of VR-1 activation, reversed the RK-induced increase in CGRP release from DRG (Fig. 3).

3.2. Effect of topical application of RK on dermal IGF-I levels in WT and CGRP−/− mice

When applied topically, 0.005% and 0.01% RK significantly increased dermal IGF-I levels at 30 min after application in WT mice ($p < 0.01$) (Fig. 4). However, dermal IGF-I levels were not increased at 30 min after

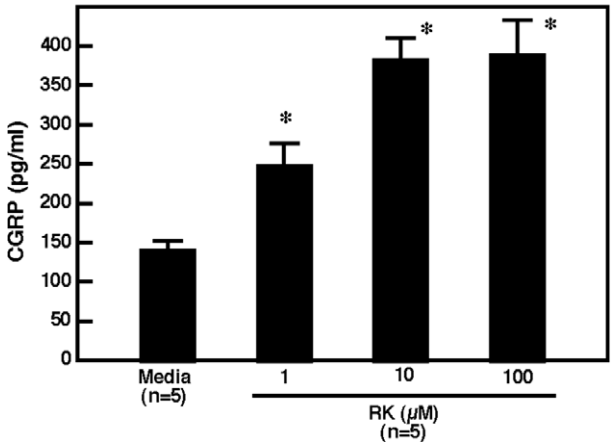

Fig. 2. Effects of raspberry ketone (RK) on CGRP release from DRG isolated from WT mice. DRG neurons were incubated with RK (1, 10, and 100 μM) for 30 min. Supernatants were collected, and CGRP levels were measured by enzyme immunoassay. Each value represents the mean ± SD from 5 experiments. *, $p < 0.01$ vs. media.

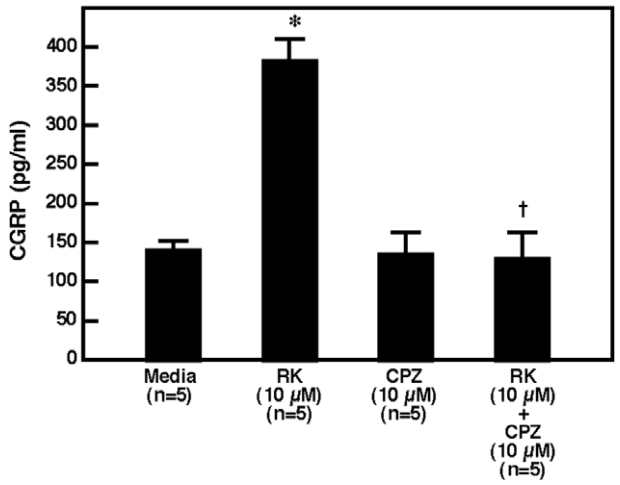

Fig. 3. Effects of RK and/or capsazepine (CPZ) on CGRP release from DRG isolated from WT mice. DRG neurons were incubated with RK (10 μM) and/or CPZ (10 μM), an inhibitor of vanilloid receptor-1 activation, for 30 min. Each value represents the mean ± SD from 5 experiments. *, $p < 0.01$ vs. media. †, $p < 0.01$ vs. RK.

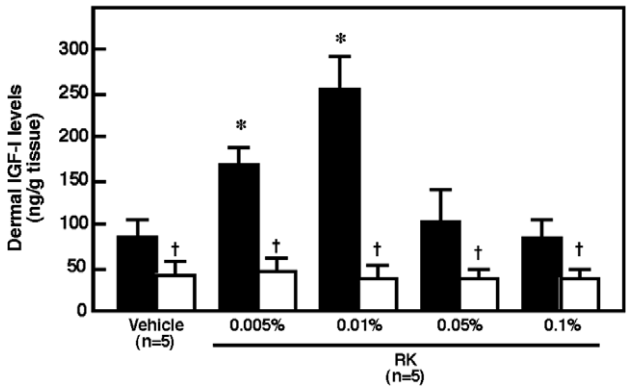

Fig. 4. Effect of topical application of RK on dermal IGF-I levels in WT and CGRP−/− mice. Skin samples were dissected 30 min after topical application of RK. Each value represents the mean ± SD from 5 experiments. Closed bar, WT mice; open bar, CGRP−/− mice. *, $p < 0.01$ vs. vehicle. †, $p < 0.01$ vs. WT.

topical application of either 0.05% or 0.1% RK (Fig. 4). Although topical application of 0.01% RK increased dermal IGF-I levels in WT mice, it had no such effects in CGRP−/− mice (Fig. 4). Concentrations of RK in the skin 30 min after topical application of 0.01% and 0.1% RK were 32.33 ± 2.25 μM ($n = 3$) and 300.12 ± 23.53 μM ($n = 3$), respectively.

3.3. Effect of topical application of 0.01% RK on dermal IGF-I levels and immunohistochemical expression of IGF-I at dermal papillae in hair follicles of WT mice

Dermal IGF-I levels were more significantly increased in WT mice to which 0.01% RK had been topically applied for 4 weeks than in WT mice receiving vehicles for 4 weeks (Fig. 5). Immunohistochemical expression of IGF-I at dermal papillae in hair follicles was clearly higher in WT mice to which 0.01% RK had been topically applied for 4 weeks (Fig. 5, inset B) than in those receiving vehicles for 4 weeks (Fig. 5, inset A).

3.4. Effect of RK on hair re-growth in WT mice

Hair re-growth in WT mice at 4 weeks after hair removal was promoted more in WT mice with 0.01%RK topically applied for 4 weeks (Fig. 6B) than in those with vehicles topically applied for 4 weeks (Fig. 6A).

3.5. Effect of topical application of RK on hair growth in volunteers with alopecia

We examined the effect of topical application of 0.01% RK to the scalp on hair growth in 10 volunteers with alopecia. Five of the volunteers (5/10; 50.0%) with alopecia showed promotion of hair growth at 5 months after topical application of 0.01% RK. Representative effects of this topical application of 0.01% RK in 4 volunteers with alopecia are shown in Fig. 7. Hair growth was observed at 5 months after topical application of 0.01% RK in male volunteers with AGA (Fig. 7A–C) and in a female volunteer with AA (Fig. 7D).

3.6. Effects of topical application of 0.01% RK and vehicle on cheek skin elasticity in 10 healthy female volunteers

When applied topically to the facial skin of 5 healthy female volunteers once a day for 14 days, 0.01% RK significantly increased cheek skin elasticity as compared with that before topical application ($p < 0.04$) (Fig. 8A). Topical application of vehicles did not increase cheek skin elasticity in the other 5 healthy female volunteers (Fig. 8B).

4. Discussion

In the present study, we examined whether RK activates sensory neurons, thereby increasing CGRP release from sensory neurons isolated from WT mice. Since the structure of RK is quite similar to that of capsaicin, it is possible that RK activates sensory neurons by activating VR-1. Consistent with this hypothesis are observations in the present study demonstrating that RK, at concentrations higher than 1 μM, increased CGRP release from DRG isolated from WT mice and that this increase was completely reversed by CPZ, an inhibitor of VR-1 activation.

We previously reported that activation of sensory neurons by topical application of capsaicin increases dermal IGF-I production in WT mice [15], suggesting

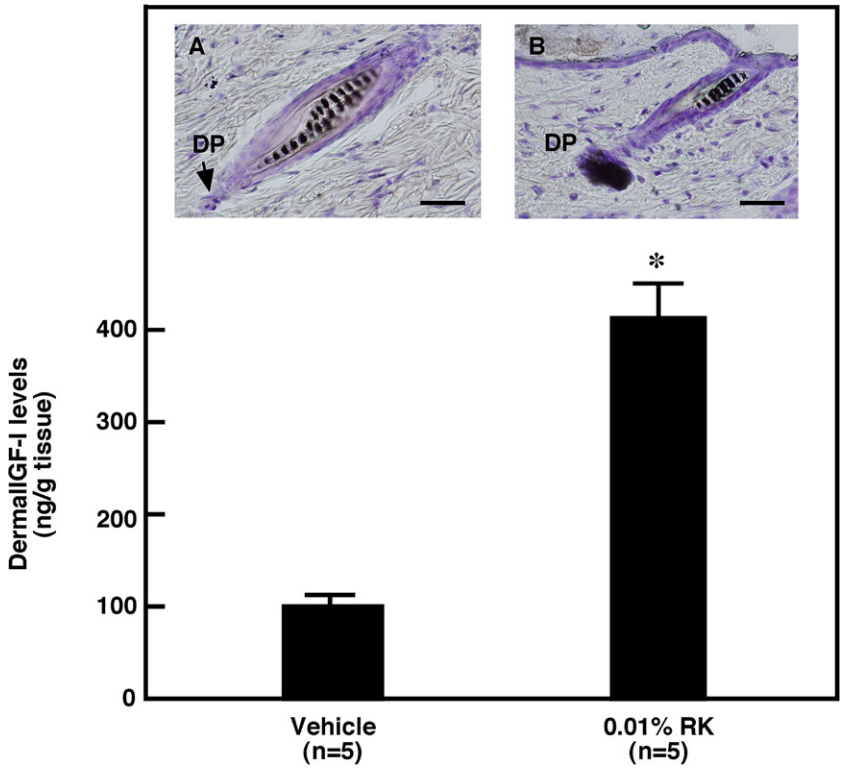

Fig. 5. Effect of topical application of 0.01% RK on dermal levels of IGF-I and immunohistochemical expression of IGF-I at dermal papillae in hair follicles in WT mice. Skin samples were dissected at 4 weeks after topical application of 0.01% RK. Each value represents the mean ± SD from 5 experiments. *, $p < 0.01$ vs. vehicle. Immunohistochemical expression of IGF-I in the skin is shown in insets: (A) vehicle; (B) 0.01% RK. Dermal papillae in hair follicles are indicated by DP. Typical results are shown in the photographs from 5 experiments. Scale bars = 50 μm.

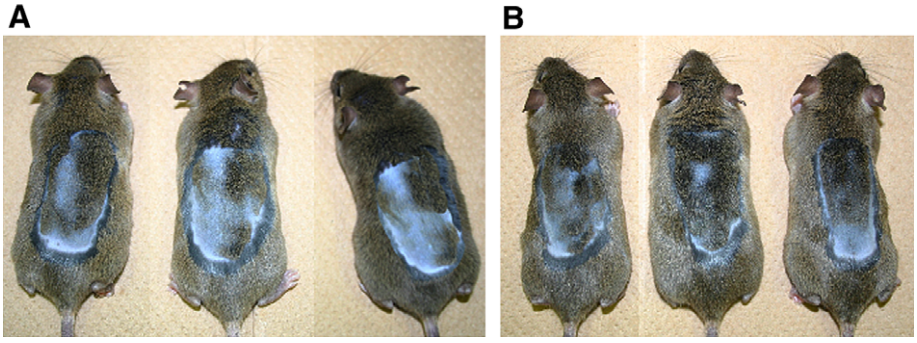

Fig. 6. Effect of RK on hair re-growth in WT mice. Hair re-growth in WT mice was shown at 4 weeks after hair removal and topical application of 0.01% RK: (A) Vehicle; (B) 0.01% RK.

that topical application of RK might increase dermal IGF-I levels by activating sensory neurons in WT mice. Consistent with this hypothesis are observations in the present study showing that topical application of 0.005% and 0.01% RK increased dermal IGF-I levels in WT mice, but not in CGRP−/− mice at 30 min after topical application. Since skin fibroblasts have been shown to express the CGRP receptor [24] and are capable of producing IGF-I [8], our present observations suggest that topical application of 0.01% RK might increase dermal IGF-I levels in skin fibroblasts through activation of sensory neurons.

Although RK increased CGRP release from DRG isolated from WT mice in a concentration dependent manner *in vitro*, RK, at concentrations higher than 0.05%, did not increase dermal IGF-I levels in these mice at 30 min after topical application. We previously reported that dermal IGF-I levels increased transiently after topical application of 0.01% capsaicin in WT mice [15]. Preliminary experiments showed that, although dermal IGF-I levels at 30 min after topical application of 0.05% and 0.1% capsaicin were significantly lower than those after topical application of 0.01% capsaicin, at 15 min after 0.05% and 0.1% capsaicin application,

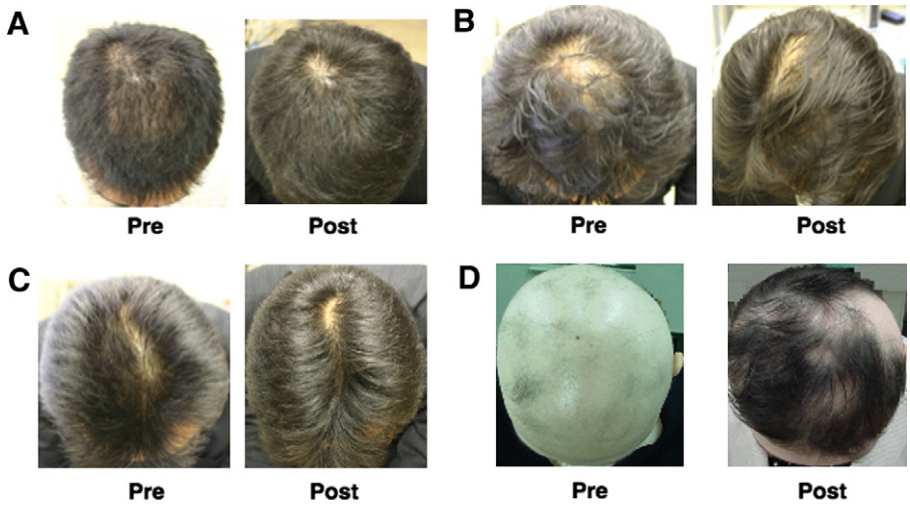

Fig. 7. Effect of topical application of RK on hair growth in volunteers with alopecia. Hair growth was observed at 5 months after topical application of 0.01% RK in 3 males volunteer with AGA (A, 30 years old; B, 28 years old; C, 36 years old) and in a female volunteer with AA (D, 27 years old).

these levels were significantly higher than those after 0.01% capsaicin. These observations suggest that 0.05% and 0.1% RK might more strongly stimulate sensory neurons than 0.005% and 0.01% RK, thereby rapidly increasing dermal IGF-I levels with peak time points earlier than 30 min after topical application and that these increases might be followed by a rapid decrease due to depletion of CGRP from sensory neurons. Consistent with this hypothesis are observations made in the present study demonstrating that RK concentrations in the skin at 30 min after topical application of 0.1% RK were 10 times higher than those after 0.01% RK application. Thus, excessive stimulation of sensory neurons by high concentrations of RK might explain why 0.01% RK increased dermal IGF-I levels, while neither 0.05% nor 0.1% RK had this effect 30 min after topical application as shown in the present study. This possibility should be further investigated.

Topical application of 0.01% RK increased dermal IGF-I levels at 30 min after application as shown in the present study. Production of IGF-I via capsaicin *in vivo* is more rapid than that occurring in response to CGRP *in vitro* [25], indicating that the former might not be mediated by the increase in transcription as in the latter, but rather by other unknown mechanism(s). Our previous report demonstrated that subcutaneous administration of capsaicin increased tissue levels of IGF-I and IGF-I mRNA in various organs including the skin at 30 min after administration in mice [14], raising the possibility that stimulation of sensory neurons by topical application of 0.01% RK might increase IGF-I production by increasing its transcription. This possibility should be examined in future experiments.

In the present study, immunohistochemical expression of IGF-I at dermal papillae in hair follicles was clearly increased in WT mice to which 0.01% RK had been topically applied for 4 weeks as shown in the present study. Consistent with these observations

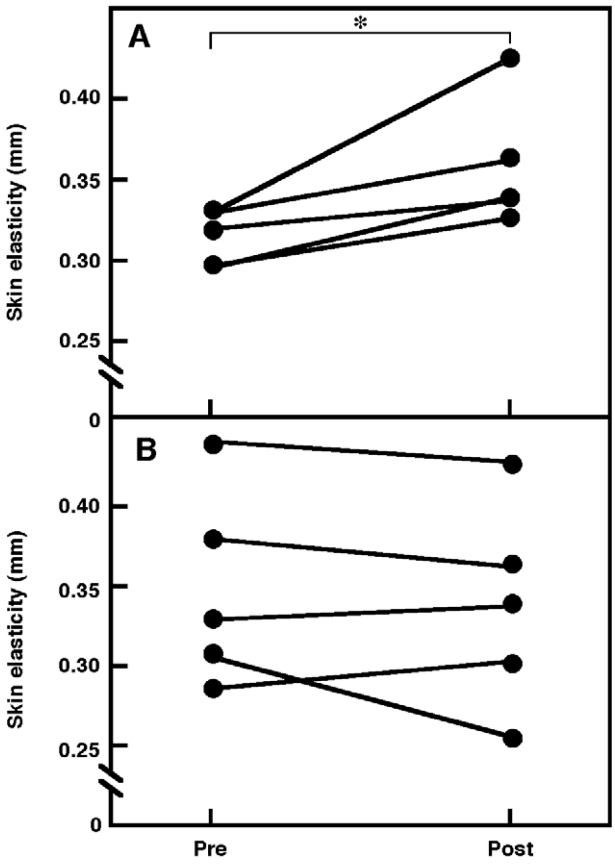

Fig. 8. Effect of topical application of 0.01% RK (A) or vehicle (B) on cheek skin elasticity in 10 healthy female volunteers. RK or vehicle was applied to facial skin of each 5 female volunteers once a day for 14 days. Cheek skin elasticity was measured by Cutemeter before and after experiments. *, $p < 0.04$ vs. pre.

is a previous report demonstrating that IGF-I is produced by dermal papilla cells [7]. IGF-I is known to be an important growth factor in many biological systems [26] and it has also been shown to play a critical role in promoting hair growth [4]. Since IGF-I receptor mRNA was detected in keratinocytes [8], it is possible that IGF-I produced by dermal papilla cells acts on keratinocytes, thereby promoting hair growth through stimulation of the proliferation of keratinocytes in hair follicles [4]. Thus, it is possible that increase in IGF-I levels in hair follicles with topical application of 0.01% RK promotes hair growth in WT mice. Consistent with this hypothesis are our present observations demonstrating that hair re-growth was more marked in WT mice to which 0.01% RK had been topically applied for 4 weeks than in those receiving vehicles for 4 weeks.

In contrast to our results, capsaicin has been shown to inhibit hair shaft elongation by inducing premature hair follicle regression via VR-1 stimulation of the outer root sheath keratinocytes in organ-cultured human scalp hair follicles [27]. However, in the present study, RK significantly increased IGF-I expression and promoted hair re-growth in mice. These observations suggest that RK might interact mainly with VR-1 on sensory neurons, thereby increasing IGF-I production in dermal papillae through an increase in CGRP release from sensory neurons *in vivo*.

Topical application of 0.01% RK to the scalp for 5 months promoted hair growth in 5 of the 10 volunteers with alopecia in the present study. Consistent with these observations is our recent report demonstrating that administration of capsaicin and isoflavone promoted hair growth by increasing IGF-I production in humans with alopecia [19]. These observations raised the possibility that topical application of 0.01% RK to the scalp increases IGF-I production in hair follicles through activation of sensory neurons, thereby promoting hair growth in humans suffering from alopecia. These possibilities should be further examined in a large controlled study of human subjects with alopecia.

We previously demonstrated that stimulation of sensory neurons increases tissue blood flow by increasing endothelial productions of nitric oxide and prostacyclin through activation of endothelial nitric oxide synthase and cyclooxygenase-1, respectively [28]. Thus, it is possible that stimulation of sensory neurons by RK increases dermal blood flow, thereby contributing to the promotion of hair growth. Furthermore, capsaicin has been shown to down-regulate androgen receptor expression by prostate cancer cells [29]. Since androgen plays a critical role in the development of alopecia [30] and balding hair follicle dermal papilla cells contain higher levels of androgen receptors than those from non-balding scalp [31], RK, like capsaicin, might promote hair growth by decreasing androgen action through androgen receptor down-regulation on dermal papilla cells.

CGRP has been shown to promote proliferation of human keratinocytes by increasing intracellular cAMP levels *in vitro* [32], suggesting that topical application of 0.01% RK might promote hair growth not only by increasing IGF-I production, but also by increasing CGRP release from sensory neurons in mice and humans with alopecia.

IGF-I increases the production of collagen [10] and elastin [33] by skin fibroblasts and promotes proliferation of keratinocytes [34], suggesting that IGF-I produced by fibroblasts might act on these fibroblasts themselves as well as on keratinocytes, thereby promoting the productions of both collagen and elastin as well as proliferation, respectively. Patients with Laron syndrome showed skin morphological changes such as decreased thickness with decreased elastin contents [35]. The IGF-I receptor has been demonstrated in human skin biopsies [8]. These observations suggest that detrimental skin morphological changes observed in patients with Laron syndrome might be attributable to reduced production of IGF-I.

Topical application of 0.01% RK to facial skin significantly increased cheek skin elasticity in 5 healthy female volunteers after 14 days of application ($p < 0.04$). Consistent with these observations is our previous report [15] demonstrating that topical application of 0.01% capsaicin to facial skin increased cheek skin elasticity in female volunteers. The important mechanical property that primarily maintains skin elasticity is attributable to the content and molecular structure of collagen fibers embedded in the ground substance [36]. The sweat secretion rate has been found to be decreased in patients with GH deficiency who have low serum IGF-I levels [37]. Decreased ability to sweat results from the atrophy of eccrine sweat glands due to lack of stimulation by either GH or IGF-I, or both [38]. Since intra-epidermal elasticity is known to be associated with the presence of sweat [39], topical application of 0.01% RK might have increased sweat in the facial skin epidermis of the volunteers, thereby contributing to the increase in facial skin elasticity.

Detrimental skin morphological changes such as decreases in skin thickness and collagen contents are observed in postmenopausal women [40] as well as in patients with Laron syndrome [35], raising the possibility that topical application of 0.01% RK might increase skin elasticity, probably by increasing dermal IGF-I contents in aged women.

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