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ORIGINAL ARTICLE

EXPRESSION OF Raf KINASE INHIBITOR PROTEIN IN KERATINOCYTE DIFFERENTIATION IN THE MOUSE EPIDERMIS

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Abstract Raf kinase inhibitor protein (RKIP) has been demonstrated to bind to Raf-1 and mitogen-activated protein kinase kinase (MEK) and to inhibit the mitogen-activated protein kinase (MAPK) signaling pathway. Recently, we have discovered that introduction of the RKIP gene into human keratinocytes induces differentiation. In this study, we first examined expression of RKIP in the mouse epidermis in vivo and found that RKIP was expressed in all keratinocyte layers. However, western blot analysis clearly showed that differentiated keratinocytes expressed more RKIP than undifferentiated keratinocytes. These data indicated that RKIP expression in the mouse epidermis increased with keratinocyte differentiation. As 1α,25-dihydoxyvitamin D_3 ($1,25(OH)_2D_3$) suppresses keratinocyte proliferation and promotes differentiation, we next examined the effect of $1,25(OH)_2D_3$ on RKIP expression and found that $1,25(OH)_2D_3$ increased mRNA and protein expression of RKIP in cultured mouse keratinocytes. These results suggest that $1,25(OH)_2D_3$-induced keratinocyte differentiation may be mediated by induction of RKIP expression.

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Key words: keratinocytes; signal transduction; differentiation; vitamin D.

原　著

マウス表皮での表皮角化細胞分化における Raf kinase inhibitor protein の発現

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抄録 Raf kinase inhibitor protein (RKIP) は Raf-1 や mitogen-activated protein kinase kinase (MEK) に結合して，mitogen-activated protein kinase (MAPK) シグナル伝達を抑制することが明らかにされている．近年，我々は RKIP 遺伝子をヒト表皮角化細胞に導入することにより，表皮角化細胞の分化を誘導できることを示した．本研究では，まず，マウス表皮での RKIP の発現を検討した．その結果，マウスの表皮では全層で RKIP の発現が認められた．しかしながら，培養表皮細胞を用いた実験で，より分化した表皮角化細胞は未分化状態の細胞に比べて，RKIPを強く発現することを示すことができた．この結果は，マウスの表皮でも RKIP 発現が表皮角化細胞の分化に伴って増加することが明らかにされた．1α,25-dihydoxyvitamin D_3 ($1,25(OH)_2D_3$) は表皮角化細胞の増殖を抑制し，分化を誘導することが知られている．そこで，次に $1,25(OH)_2D_3$ の RKIP に対する発現を検討した．その結果，マウス培養表皮角化細胞において $1,25(OH)_2D_3$ は RKIPの mRNA と蛋白をともに上昇することが示された．この結果，$1,25(OH)_2D_3$ が RKIP の発現誘導を介してマウスの表皮角化細胞の分化を誘導する可能性が示唆された．

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Introduction

Raf kinase inhibitor protein (RKIP), a member of the phosphatidylethanolamine-binding protein (PEBP) family, is a small cytosolic protein that is highly conserved among many mammalian species. While RKIP was originally purified from the bovine brain, it is expressed in a wide variety of tissues[1]. RKIP has been shown to interact with the classical mitogen-activated protein kinase (MAPK) signaling pathway. Yeung et al. demonstrated that RKIP could bind to Raf-1 and MEK-1 and weakly to ERK-2, interfering with MEK phosphorylation and activation by Raf-1[2] and subsequently inhibiting downstream MAPK signaling. RKIP also inhibits cell growth by interacting with protein kinase C (PKC), which phosphorylates regulatory proteins involved in cell growth and differentiation. Corbit et al. revealed that PKC phosphorylates RKIP on serine 153 and rescues ERK inhibition by RKIP [3].

RKIP is expressed in many tissues, including the testis, adrenal gland, liver, and kidney, suggesting that the protein has an important and conserved biological function. However, the exact functions of RKIP in most tissues remain unknown. Our group has recently demonstrated that RKIP induces differentiation of human epidermal keratinocytes. Transient expression of RKIP in HaCaT cells, an immortalized human epidermal keratinocyte cell line, resulted in increased expression of involucrin and morphological changes, both characteristic of terminal keratinocyte differentiation[4].

Recently, 1α,25-dihydoxyvitamin D_3 (1,25 $(OH)_2D_3$) has been shown to stimulate differentiation in epidermal keratinocytes, and topical application of 1,25$(OH)_2D_3$ is widely used in clinical dermatology for the treatment of psoriasis[5]. However, the inhibitory mechanism of 1,25$(OH)_2D_3$ in keratinocyte differentiation is not yet fully understood. The hormone is known to bind to the vitamin D3 receptor, and the complex then acts as a transcriptional factor that modulates the expression of various genes possessing vitamin D response elements. Recent studies, however, have revealed other regulatory mechanisms for 1,25$(OH)_2D_3$ that are mediated through the activation of MAPK signaling[6]. Therefore, we hypothesized that 1,25 $(OH)_2D_3$ induced keratinocyte differentiation in part by modulating RKIP expression. In this study, we examined the effect of 1,25$(OH)_2D_3$ on RKIP expression in mouse epidermal systems.

Materials and Methods

Cell culture

Newborn BALB/c mice (0–3 days) were killed on ice and the trunk skin was removed. The skins were incubated overnight at 4°C in dispase. The epidermis was separated from the dermis and incubated at 37°C in trypsin for 5 min. The epidermal sheets were removed and the cells were collected by centrifugation. The keratinocytes were suspended in complete keratinocyte growth medium and seeded in fibronectin-collagen precoated plates. The medium was prepared by adding $CaCl_2$ to a final concentration of 0.05 mM to minimum essential medium (without $CaCl_2$, IWAKI) containing 8% fetal bovine serum pretreated with Chelex resin (BioRad). The growth medium was supplemented with an antibiotic mixture (penicillin, streptomycin sulfate, amphotericin B; Life Technologies, Rockville) and used to incubate fibroblasts for 48 h. The fibroblast-conditioned medium was then filtered and used as a complete keratinocyte growth medium. Culture medium was exchanged after 24 h and every 2–3 days thereafter. Cells were maintained in monolayer cultures at 37°C containing 5% CO_2.

Western blot

Keratinocytes were harvested and incubated

on ice in reporter lysis buffer (Promega). The lysates were centrifuged for 15 min at 12,000 rpm, and the supernatant fractions were stored at –80°C. Protein concentrations were determined by the Lowry method. Lysate samples containing 35–50 μg of protein were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on 12.5% (w/v) gels and electroblotted onto Hybond nitrocellulose membranes. Blots were probed with anti-RKIP antibody followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000, v/v; Bio-Rad)[4]. Enhanced chemiluminescence (Amersham Pharmacia Biotech) was used for detection.

Immunohistochemistry

Mouse skin sections were fixed in 10% formaldehyde and embedded in paraffin. The sections were routinely passed through xylene and a graded alcohol series and stained with anti-RKIP antibody by the avidin-biotin-peroxidase complex (ABC) method [4]. Peroxidase binding was detected by the diaminobenzidine method, and the sections were then lightly counterstained with hematoxylin for microscopic examination. The experiment was performed in accordance with Guidelines for Animal Experimentation, Hirosaki University.

Northern blot

Total RNA was extracted from cultured mouse keratinocytes using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Fifteen micrograms of total RNA was resolved by electrophoresis and transferred to nitrocellulose. The filters were hybridized with ^{32}P-labeled cDNA probes and exposed to Kodak XAR-5 film at –80°C. The cDNA probes for RKIP, involucrin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were generated with the reverse transcription-polymerase chain reaction (RT-PCR) kit (RevarTra, TOYOBO), and the cDNA was reverse-transcribed from total RNA isolated from cultured mouse keratinocytes. The 5′-primer 5′-GGCACGAGCAACTCTCCATCG-3′ and the 3′-primer 5′-GAAGACACCCTAAACCTCTAT-3′ were used to amplify mouse RKIP from the cDNA. The nucleotide sequences of these cDNAs were confirmed by sequencing with a BigDye Terminator kit (PE Biosystems, Forester City, CA) and an ABI DNA sequencer Model 310 (PE Biosystems). Quantification of the results was performed by scanning the film with Photoshop version 12.03 software (Adobe Systems Inc.) followed by densitometry with NIH Image.

Results

RKIP expression in mouse skin keratinocytes in vivo

RKIP expression in normal mouse skin was examined by immunohistochemistry. The typical staining pattern for RKIP is shown in Fig. 1A. RKIP staining was basically seen in the nucleus and cytoplasm in all cell layers. The upper epidermis was more strongly immunoreactive than the lower epidermis in some areas of the sections.

RKIP expression in keratinocytes differentiated by calcium

To examine the relationship between RKIP expression and cell differentiation in mouse keratinocytes in vitro, mouse keratinocytes were treated with 2.0 mM $CaCl_2$ to induce keratinocyte differentiation. On microscopic examination, the cells incubated with 0.05 mM $CaCl_2$ for 48 h retained elongated shapes and loose arrangement, while the cells incubated with 2.0 mM $CaCl_2$ exhibited polygonal shapes and dense packing. Western blot analysis revealed that RKIP protein levels were increased in cells treated with a higher concentration of calcium compared with those

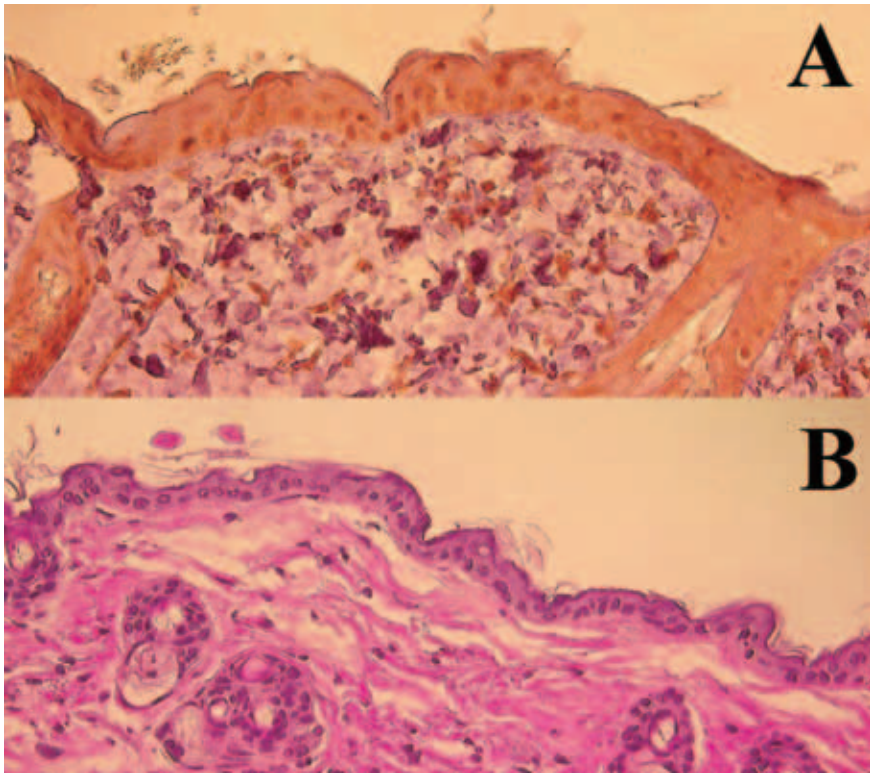

Figure 1 Immunohistochemical analysis of RKIP expression in the mouse skin.
RKIP expression in normal mouse skin was examined by immunohistochemistry (A). Peroxidase binding was detected by the diaminobenzidine method. The skin sections were stained with hematoxylin and eosin (B). RKIP staining was found in the nucleus and cytoplasm in all cell layers. The upper epidermis was more strongly immunoreactive than the lower epidermis. Original magnification, ×200.

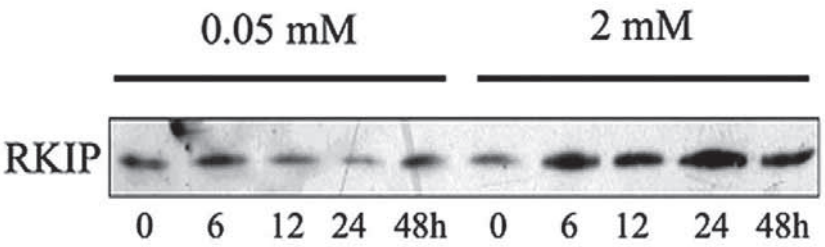

Figure 2 Effects of a high calcium concentration on RKIP expression in normal mouse epidermal keratinocytes.
Normal mouse epidermal keratinocytes were cultured for 6, 12, 24, or 48 h with 0.05 mM or 2.0 mM calcium. Each lane contains 35 μg of protein, and western blot analysis was performed as described in the Materials and Methods. RKIP protein levels were increased in cells treated with a higher concentration of calcium compared with those treated with a lower concentration.

treated with a lower concentration (Fig. 2).

RKIP expression with keratinocyte differentiation induced by 1,25-dihydroxyvitamin D3

In order to examine the relationship between $1,25(OH)_2D_3$ and RKIP expression, mouse keratinocytes were treated with various concentrations of $1,25(OH)_2D_3$. RKIP levels increased with $1,25(OH)_2D_3$ treatment in a dose-dependent manner (Fig. 3). Next, we examined $1,25(OH)_2$

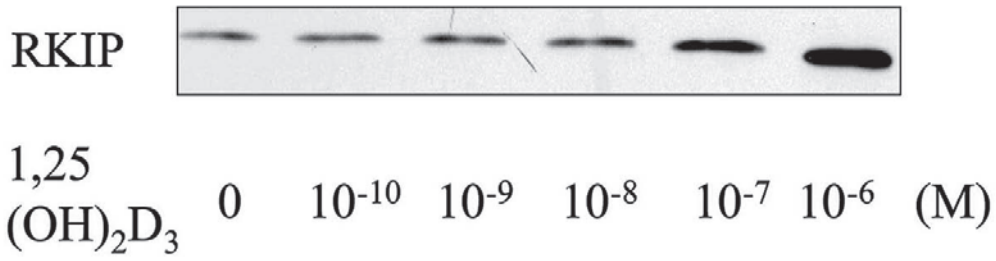

Figure 3 Effects of $1,25(OH)_2D_3$ on RKIP expression in normal mouse epidermal keratinocytes.
Normal mouse epidermal keratinocytes were cultured for 48 h with various concentrations of $1,25(OH)_2D_3$. Each lane contains 35 μg of protein, and western blot analysis using an anti-RKIP antibody was performed as described in the Materials and Methods. RKIP levels increased with $1,25(OH)_2D_3$ treatment in a dose-dependent manner.

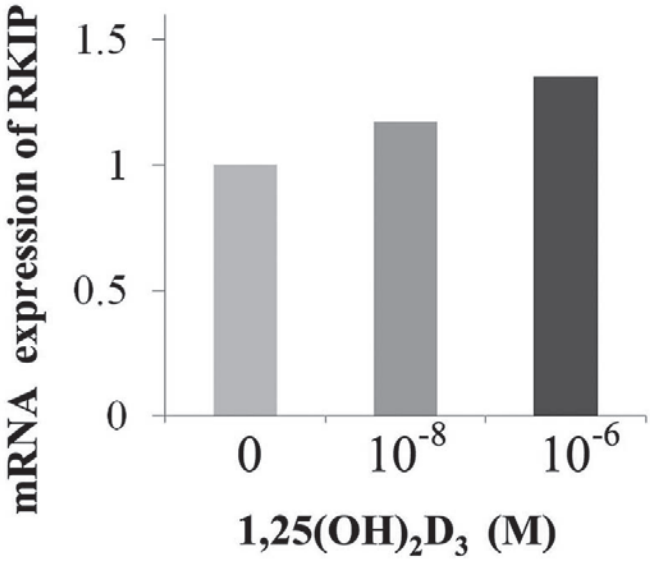

Figure 4 Effects of $1,25(OH)_2D_3$ on RKIP mRNA expression in normal mouse epidermal keratinocytes.
Total RNA was extracted from mouse epidermal keratinocytes cultured for up to 24 h with $1,25(OH)_2D_3$ at concentrations of 10^{-6} and 10^{-8} M. Fifteen micrograms of total RNA from each sample was subjected to northern blotting as described in Materials and Methods. The RKIP mRNA levels are expressed relative to GAPDH levels under the same conditions. RKIP mRNA expression also increased with increasing concentrations of $1,25(OH)_2D_3$.

D_3-induced changes in RKIP mRNA expression. The cells were treated with $1,25(OH)_2D_3$ at concentrations of 10^{-6} and 10^{-8} M and northern blot analysis was performed. RKIP mRNA levels are expressed as values relative to GAPDH levels (Fig. 4). RKIP mRNA expression also increased with increasing concentrations of 1,25 $(OH)_2D_3$.

Discussion

Keratinocytes move from the basement membrane zone towards the skin surface, forming several well-defined layers during transit. Those layers are, in ascending order, the basal layer, spinous layer, granular layer, and cornified layer. The classical MAPK signaling

pathway, composed of Raf, MEK, and ERK, stimulates cell proliferation in a variety of cell types[7]. High calcium concentrations are known to induce cell cycle arrest and differentiation in normal human keratinocytes by blocking the ERK pathway[4]. To demonstrate RKIP expression in mouse skin, we first performed immunohistochemical analysis and found that RKIP was expressed in all layers of the mouse epidermis (Fig. 1), although the upper epidermis showed stronger immunoreactivity than the lower epidermis in some areas of the sections. We have previously shown that in normal human skin, RKIP is expressed in the spinous and granular layers but not in the basal layer of the epidermis. The mouse epidermis is much thinner than the human and consists of only 2–3 layers of keratinocytes, so we could not confirm a clear relationship between RKIP expression and keratinocyte differentiation by this method. Therefore, we next examined RKIP expression in mouse keratinocytes following calcium-induced differentiation. Western blot analysis clearly showed that differentiated keratinocytes expressed more RKIP than did undifferentiated keratinocytes. These data indicated that in the mouse epidermis, RKIP expression increases with keratinocyte differentiation.

1α,25-Dihydoxyvitamin D_3 ($1,25(OH)_2D_3$) is the active form of vitamin D_3. It plays roles in many biological processes, including calcium homeostasis and bone formation. $1,25(OH)_2D_3$ binds to and activates its nuclear receptor, the vitamin D receptor (VDR), which subsequently modulates physiological events such as cellular proliferation and differentiation. Keratinocytes express receptors for vitamin D_3, and under low calcium conditions, $1,25(OH)_2D_3$ suppresses keratinocyte proliferation and promotes differentiation in a concentration-dependent manner[8]. The active vitamin D_3 analogs tacalcitol, calcipotriol, and maxacalcitol also suppress and stimulate keratinocyte proliferation and differentiation, with similar potencies to vitamin D_3 itself[9]. The effects of $1,25(OH)_2D_3$ are mediated through a nuclear protein, VDR. Following $1,25(OH)_2D_3$ binding, VDR forms homodimers or heterodimers with 2 other nuclear receptors, RXR and the thyroid hormone receptor, and the complexes bind to a specific DNA consensus sequence that includes AGGTCA repeats, inducing the expression of various vitamin D_3-dependent genes. This is referred to as the genomic response[10]. However, a non-genomic mechanism associated with rapid hormone regulation of signal transduction pathways has also been demonstrated[11]. Recent studies have revealed that $1,25(OH)_2D_3$ modulates the MAPK signaling pathway in various cell lines[6, 12]. It has also been reported that VDR-knockout mice show no remarkable alteration in keratinocyte differentiation except for alopecia[13]. In this study, we showed that $1,25(OH)_2D_3$ increased mRNA and protein expression of RKIP. These results suggest that $1,25(OH)_2D_3$ affects keratinocyte differentiation through the non-genomic mechanism. $1,25(OH)_2D_3$- induced expression of RKIP may play an important role in the differentiation of normal mouse epidermis. Further studies are needed to clarify the detailed mechanisms of $1,25(OH)_2D_3$ induction of keratinocyte differentiation.

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