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Epidermis Proliferative Effect of the *Panax ginseng* Ginsenoside Rb_2

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Ginseng has been used as a traditional medicine with various therapeutic effects. However, it is still unknown which component of this plant is effective at promoting wound healing. Recently, ginsenoside Rb_2 has been reported to improve wound healing. In this study, to investigate the reported wound healing effect of the ginsenoside Rb_2, cell morphology and protein factors involved in epidermal formation were evaluated by immunochemical and immunoblotting analysis. Rb_2 stimulated epidermal cell proliferation, and the cell showed a 1.5-fold increase in thymidine uptake compared to the control ($p<0.05$, $n=3$). Furtheremore, Rb_2 was found to stimulate epidermis formation in a dose-dependent manner in raft culture, and to dose dependently enhance the expressions of protein factors related to cell proliferation, namely, epidermal growth factor and its receptor, fibronectin and its receptor, keratin 5/14, and collagenase I ($p<0.05$, $n=3\sim9$). It is believed that ginsenoside Rb_2 enhances epidermal cell proliferation by upregulating the expressions of these proliferation-related factors.

Key words: *Panax ginseng*, Ginsenoside Rb_2, Epidermal growth factor, Fibronectin, Keratin, Collagenase

INTRODUCTION

Ginseng is reported to have therapeutic properties against a number of diseases, such as atherosclerosis, cerebrovascular diseases, liver dysfunction, and hypertension (Yamamoto *et al.*, 1988). Morisaki *et al.* (1995) found that Rb_2 stimulated wound healing with enhanced angiogenesis *in vivo*. Moreover, in an *in vivo* animal model, the local administration of ginsenoside Rb_2 markedly improved wound healing (Kanzaki *et al.*, 1998). Therefore, it was aimed to examine effects of Rb_2 upon epidermal cell proliferation and to elucidate its functional mechanism.

The wound healing process can be considered to be composed of three overlapping events, inflammation, new tissue formation, and matrix remodeling (Dunphy, 1974). In the case of new tissue formation, epidermal growth factors (EGF) and their receptors (EGFR) are involved in signal transmission. Moreover, the keratins, especially keratin 5/14 (K5/14), are involved in the formation of the cytoskeleton, and fibronectin (Fn) and its receptor (FnR) in cell locomotion, and collagenase in cell detachment. This study was undertaken to determine whether Rb_2 stimulates epidermis cell proliferation, and if so, to identify the factors influenced by Rb_2.

In this study, a raft culture system was used. This is a three-dimensional culture designed to permit long-term culture of keratinocytes at an air-liquid interface. The process of culture at the air-liquid interface enhances the organization and differentiation of air-exposed stratified keratinocytes (Asselineau *et al.*, 1985). Thus, this system allowed the epidermis proliferative effect of Rb_2 to be examined with respect to morphological changes of keratinocytes and the related biochemical effects.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade and purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise mentioned. Ginsenoside Rb_2 and whole extract of *Panax ginseng* were purchased from Indofine Chemical Co. (Sommerville, NJ, USA).

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Thymidine uptake assay

The effect of Rb_2 upon cell proliferation was evaluated by measuring the thymidine uptake of human squamous cell carcinoma 13 (SCC13) cells in the presence of Rb_2. SCC13 cells were seeded in a 96-well tissue culture plate at 5,000 cells per well, in a medium containing Dulbecco's modified Eagle's medium (Gibco BRL, Grand island, NY, USA) and Hams nutrient (Gibco BRL) mixture (3:1 by weight), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA). When the cell population reached 80% of confluency, Rb_2 or whole ginseng extract was applied at 1 μg/ml in 100 μl ethanol with serum-free media, and incubation was continued for 24 h. The negative control was given vehicle only. [^{3}H]Thymidine (Amersham, Arlington Heights, IL, USA) was then added at 5 μCi/ml and incubated for 3 h. Unreacted thymidine was washed out with phosphate buffered saline (PBS). Cells were dried at room temperature for 15 min, and fixed in 10% trichloroacetic acid (TCA) at 4°C for 1 h. The TCA was then removed by washing with PBS. Cellular nucleic acids were dried at room temperature for 15 min, solubilized in 0.5 M NaOH for 6 h, neutralized with 0.5 M HCl, and collected and mixed with scintillation cocktail (Packard, Downers Grove, IL, USA). Thymidine uptake was measured using a scintillation counter (Packard).

Preparation of keratinocytes

Foreskin keratinocyte cultures were prepared and maintained as described by Blanton *et al.* (1989). Human foreskins were obtained from circumcised newborn babies. Foreskins were washed extensively with multiple changes of PBS, subcutaneous tissue was removed, and the remaining tissue samples were enzymatically dissociated in multiple changes of 0.25% trypsin and versene (50: 50). Epidermal sheets were peeled from the dermis, minced, and dispersed in trypsin solution. Cell suspensions were pelleted from the trypsin solution, resuspended, and washed with PBS, by centrifuging at 1,000 g for 5 min at 20°C. Cells were then raised in a tissue culture dish with dermal fibroblasts (Takara, Tokyo, Japan) as feeder layer. Cultures were maintained with a growth medium consisting of Dulbeccos modified Eagles medium and Ham's nutrient mixture F12 at a ratio of 3:1. The medium was supplemented with 10% fetal bovine serum, 1×10^{-10} M cholera toxin, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, and 2×10^{-11} M tri-iodothyronine. Cultures were fed every 3 days, subcultured by dispersing in PBS containing 0.25% trypsin and replated at a split ratio of 1:3.

Effect of Rb_2 on epidermis formation by keratinocytes in raft culture

Keratinocytes were raft cultured according to the method of Asselineau *et al.* (1985). When the cells reached 90% confluency, they were trypsinized on a collagen matrix for raft culture as follows. Mouse fibroblasts were mixed with Type-I collagen matrix (Nitta gelatin, Tokyo, Japan) at a density of 20,000 cells per Millericell, and then seeded in a 12 mm Millericel (Falcon, Lincoln Park, NJ, USA). Keratinocytes were then seeded on the matrix at 20,000 cells per Millericell. Cells were then cultured whilst submerged in media for 7 days, transferred to the air-liquid interface and then raised for 21 days. Rb_2 was applied to the cells at 1 and 100 μg/ml every two days. A portion of the epidermal tissue formed after culturing for 3 weeks was taken, fixed in Carnoy solution (ethanol:glacial acetic acid: chloroform = 6:1:3 by volume), washed with 60% and then 80% ethanol, and placed in paraffin blocks for morphological comparisons.

Immunohistochemistry and Western blotting

Slices (5 μm) of paraffin blocks were deparaffinized and hydrated before immunohistochemical staining, which was carried out as described by Choi and Fuchs (1990). Antisera and dilutions were used as follows: mouse monoclonal antisera against human EGFR, (diluted, 1:5) (Triton Diagnostics, Alameda, CA, USA); rabbit polyclonal antisera against human FnR, (1:200) (Chemicon, Temecula, CA, USA); rabbit polyclonal antisera against human Fn, (1:100) (Biomedical Tech, Stoughton, MA, USA); rabbit polyclonal antisera against human keratin 5/14, (1:100) (Chemicon). After incubating with primary antisera, the sections were subjected to Immunogold enhancement (Amersham). Immunostaining intensity was estimated using an Image Analyzer (BAS-2500, Fujifilm, Tokyo, Japan) with image analysis software (MCID ver 3.0, Imaging Research Inc., Ontario, Canada) and expressed as a percentage of the control.

To prepare whole cell lysates, keratinocytes were collected and resuspended in chilled lysis buffer (50 mM Tris-HCl pH 7.4, 1% Triton × 100, 150 mM NaCl, and 2 mM of phenylmethyl sulfonyl fluoride (PMSF)), sonicated, and centrifuged at 10,000 g for 10 min. The precipitates were then resuspended in the lysis buffer. Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Each 50 μg of SDS samples were boiled at 100°C for 5 min, loaded onto 10 % SDS PAGE gels and blotted onto PVDF membranes (Amersham). Western blotting was performed to detect EGF, EGFR, Fn, FnR, K5/14, collagenase I, and β-actin as follows: anti-EGF (1:100) (Santa Cruz Biotechnology Co., Santa Cruz, CA, USA), anti-EGFR (1:100) (Santa Cruz), anti-Fn (1:100) (Santa Cruz), anti-FnR (1:100) (Chemicon), anti-K5/14 (1:100) (Chemicon), and anti-β-actin

(1:250) (Sigma). Secondary antibody linked to horseradish peroxidase (Amersham) was diluted at 1: 6,000. Protein bands in the membrane were visualized by chemiluminescence (ECL, Amersham). Western blotting and immunostaining intensities were estimated using an Image Analyzer (BAS-2500) with image analysis software (MCID ver 3.0) and expressed as fold increases versus the respective controls.

To determine collagenase expression, keratinocytes were collected and resuspended in chilled lysis buffer (50 mM Tris-HCl pH 7.4, 1% Triton × 100, 150 mM NaCl, and 2 mM PMSF), sonicated, and centrifuged at 10,000 g for 10 min. The precipitate was then solubilized in 10% β-mercaptoethanol and solubilized in 8 M urea containing 2 mM PMSF. The homogenate was centrifuged for 5 min in a microcentrifuge at 4°C and the supernatants collected. Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad), western blotting was performed as described above with anti-collagenase I (Lab Vision Co., Fremont, CA, USA) at a dilution of 1: 250.

Statistics

The data obtained were analyzed using the Students' *t* test, and $p < 0.05$ was taken to be statistically significant.

RESULTS

Rb_2 stimulated epidermal cell proliferation

The effect of Rb_2 upon cellular proliferative activity was investigated by determining whether Rb_2 enhances [^{3}H] thymidine uptake by SCC13 cells (Fig. 1). Rb_2 was found to increase thymidine uptake about 1.5-fold that of the whole ginseng extract ($p<0.05$, $n=3$).

Rb_2 enhanced epidermis formation in raft culture

Stained paraffin sections of the artificial epidermis raised in raft culture were compared by staining with hematoxylin and eosin (Fig. 2). In the control, many basal cells died, and thin spinous, granular, and stratum corneum layers were observed. As the Rb_2 concentration was increased from 1 to 100 μg/ml, basal cells multiplied, and the number of nucleated cells increased, and a thick epithelial layer was formed. In addition, the effect of Rb_2 on the expression of protein markers related to cell proliferation was studied by immunohistochemistry (Fig. 3). Immunostaining for the EGF receptor showed that in the control only very low levels of the EGF receptor were present in the basal layer. However, as the Rb_2 concentration was increased, the receptor expression level also increased on the cell membrane of the basal layer and even in the innermost spinous cells. Consistent with the

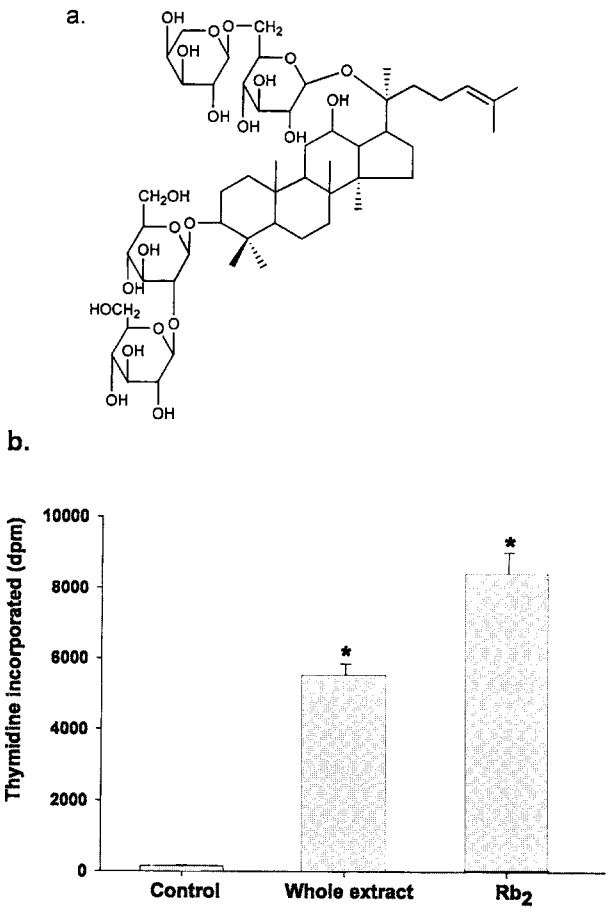

Fig. 1. Structure of ginsenoside Rb_2 (a). Effect of Rb_2 on [^{3}H] thymidine uptake by SCC13 cells (b). Rb_2 or whole ginseng extract was applied at 1 μg/ml in 100 μl ethanol. Vehicle control was given ethanol only. Data are presented as mean values ± SD ($n=3$). *Student's *t* test was used to determine the significance of difference at a *p* value of <0. 05.

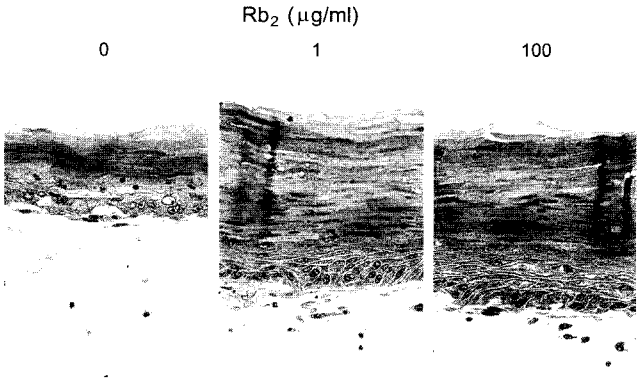

Fig. 2. Effect of Rb_2 on epidermis formation in raft cultures. Keratinocyte cultures were treated with ginsenoside Rb_2 for 21 days immediately after the air-exposed state. Cultures were grown in the absence or in the presence of 1 and 100 μg/ml Rb_2. Hematoxylin and eosin staining, original magnification × 200.

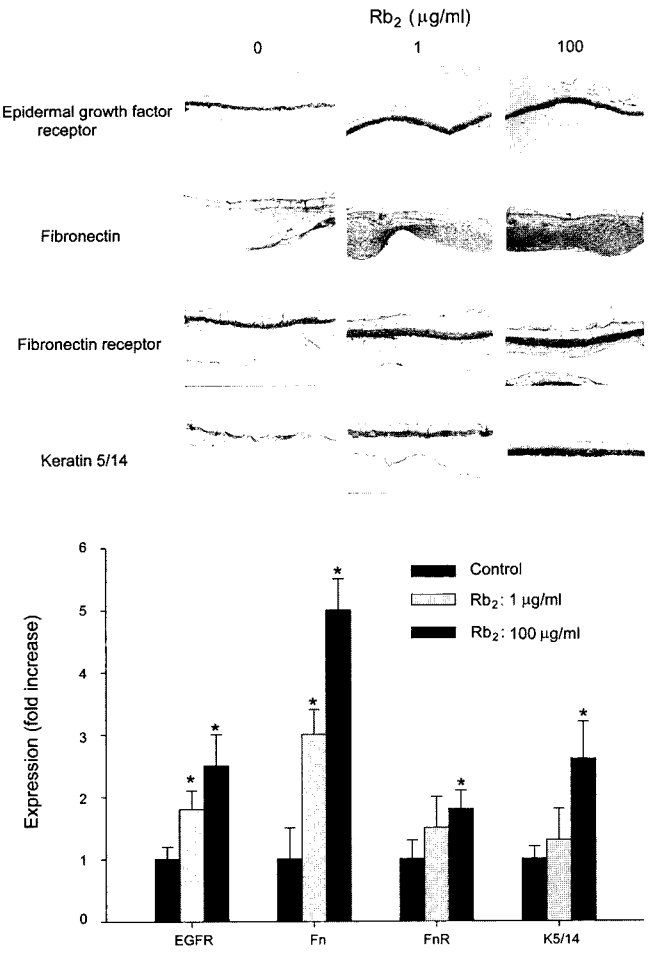

Fig. 3. Immunohistochemistry of artificial epidermis grown in raft culture. Immunohistochemical staining was used to detect EGFR, Fn, FnR, and K5/14 in artificial epidermis grown in the absence or in the presence of 1 or 100 μg/ml Rb_2. Immunostaining is indicated by black spots. Original magnification × 200. The graph below shows the relative expression levels. Intensities were determined using an Image Analyzer (BAS-2500) with image analysis software (MCID ver 3.0) and expressed as fold increases versus the respective controls. Evaluations were made in three different areas of the epidermis in three experiments. Data are presented as means ± SD (n=9), and were analyzed using the Student's t-test. *A p value of <0.05 was taken to indicate statistical significance versus the respective controls.

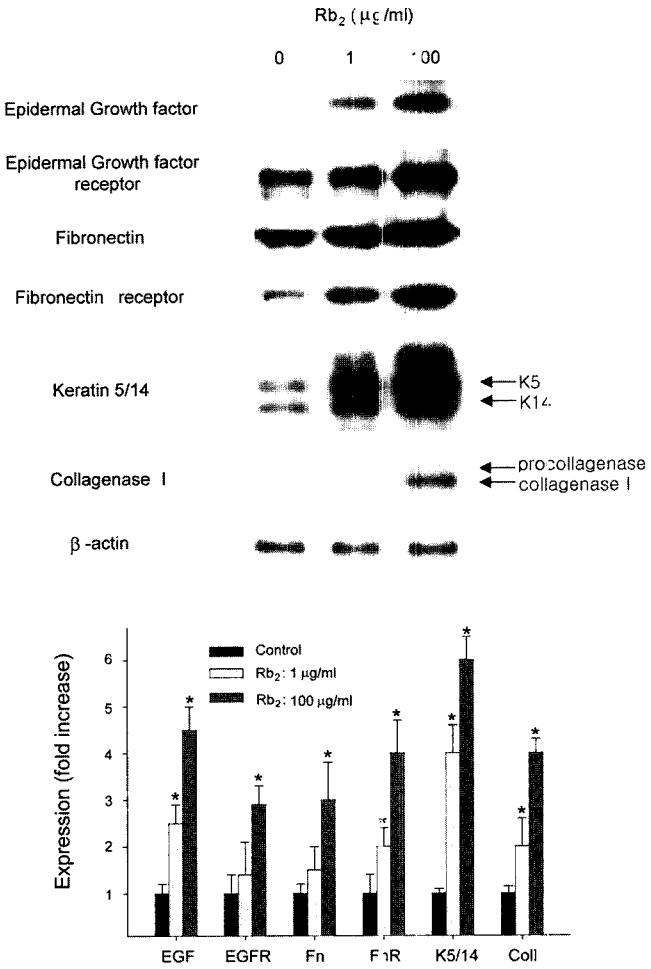

Fig. 4. The effect of Rb_2 on the expression levels of EGF, EGFR, Fn, FnR, K5/14, and Collagenase I by Western blott-ing. Rb_2 was administered at 1 and 100 μg/ml. Proteins were analyzed by immunoblotting with antibodies against EGF, EGFR, Fn, FnR, K5/14, and Collagenase I as described in Materials and Methods. β-Actin was used to confirm that the same amount of total protein was applied to each lane. The graph below shows the relative expression levels. Intensities were determined using an Image Analyzer (BAS-2500) with image analysis software (MCID ver 3.0) and expressed as fold increases versus the respective controls. Data are presented as means ± SD (n=3), and were analyzed using the Student's t-test. *A p value of <0.05 was taken to indicate statistical significance compared to the respective controls.

basal cell increases shown in the above, EGFR expression increased in a dose-dependent manner, reached 2.5-fold that of the control at 100 μg/ml of Rb_2 (p<0.05, n =9). Dermal fibroblasts embedded in the collagen matrix produced Fn, which was also induced by Rb_2 in a dose-dependent manner, for example, Fn expression increased to 5-fold that of the control at 100 mg/ml of Rb_2 (p<0.05, n =9). FnR was also found to be expressed in a dose-dependent fashion; immunostaining showed an increase up to 1.8-fold that of the control at an Rb_2 concentration of 100 μg/ml (p<0.05, n=9). Cells expressing FnR were found to be localized to the basal and suprabasal layer of the epidermis. FnR was also found between the collagen matrix and the basal layer. The expression of keratins was also examined in the artificial epidermis. Keratin 5/14 was found to be expressed in the basal and spinous layers in a dose dependent manner, and reached 2.6-fold the control level at 100 μg/ml of Rb_2 (p<0.05, n=9).

Quantitative comparisons were made of the expressions of EGF, EGFR, Fn, FnR, keratin 5/14, and collagenase I by immunoblotting using β-actin as an internal control

(Fig. 4). Compared to the control, Rb_2 treatment increased the expression of all of those proteins in a dose-dependent manner. EGF and EGFR expression increased to 4.5 and 2.9-fold that of the respective controls at 100 μg/ml of Rb_2, respectively ($p<0.05$, $n=3$). Fibroblasts embedded in the collagen matrix produced Fn, which was expressed at 3-fold that of the control at 100 μg/ml of Rb_2 ($p<0.05$, $n=3$). The expressions of FnR and of keratin 5/14 were also increased dose dependently by 4 and 6-fold that of the respective controls at 100 μg/ml of Rb_2 ($p<0.05$, $n=3$). Collagenase I was expressed at up to 4-fold that of the control in the presence of 100 μg/ml of Rb_2 ($p<0.05$, $n=3$).

DISCUSSION

Recently ginsenoside Rb_2 from *Panax ginseng* was reported to have wound healing effects (Kanzaki *et al.*, 1998). Therefore this study was undertaken to further investigate the wound healing effects of Rb_2 by examining its effects upon cell proliferation. In this study, Rb_2 stimulated the proliferation of SCC13 cells, as determined by the thymidine uptake test. At first, primary keartinocytes were tried for the thymidine uptake assay, but their growth rate was not fast enough for the assay. Thus the assay was performed using SCC13 cell only. Raft-culture and Western-blotting experiments were performed with primary keratinocytes. Rb_2 enhanced epidermis formation in raft culture, thickened the epidermal covering, produced the appearance of a proliferating phenotype and increased the expressions of EGF, EGFR, Fn, FnR, keratin 5/14, and collagenase I, which are expected to be involved in the wound healing process, in a dose-dependent fashion.

Major ginsenosides have a similar structure to cholesterol and steroid hormones (Kanzaki *et al*, 1998; Yamamoto *et al*, 1988). It is suggested that ginsenosides resembling sterols be responsible for the angiogenic activity (Morisaki *et al.*, 1995): Ginsenosides are known to help wound healing by upregulating TGF-b receptor expression and enhancing Fn synthesis (Kanzaki *et al.*, 1998). Rb_2 is reported to stimulate protein and RNA synthesis and increases RNA polymerase activity (Yokozawa *et al.*, 1993a, 1993b; Yokozawa and Oura *et al.*, 1990). There are contradictory reports concerning the cell proliferative effect of Rb_2. Mochizuki *et al.*(1995) found that Rb_2 inhibited metastasis of melanoma cell. Liu *et al.* (2000) reported upon the anti-proliferative effect of Rb_2 on human prostate cancer cell line. However, Morisaki *et al.* (1995) observed that Rb_2 stimulated wound healing with enhanced angiogenesis *in vivo*. In this study, it shows that Rb_2 has a proliferative effect on keratinocytes in raft culture. It is possible that Rb_2 increased mRNA synthesis and/or affected RNA polymerase in inducing the expression of proliferation-related factors. Why Rb_2 exerts different effects depending upon cell types is unknown and remains to be studied.

It is known that EGF, interleukin-1β, nitric oxide, and platelet-derived growth factors affect cell migration (Cumberbatch *et al.*, 1997; Nelson *et al.*, 1997; Noiri *et al.*, 1996; Nilsson *et al*, 1995). In our study, few cells survived in the absence of serum and Rb_2. However, in the presence of Rb_2, keratinocytes survived, multiplied and formed an epidermis, even in serum-free medium. In addition, Rb_2 enhanced the expressions of migration-related proteins, such as Fn, FnR, and collagenase I. It is likely that the enhancement of cell migration by Rb_2 occurred in parallel with its effect on cell proliferation.

The EGFR mediates the paracrine and autocrine growth regulation of cells (Pittelkow *et al.*, 1993). Stoscheck *et al.* (1992) demonstrated that an increase in the level of EGFR precedes hypertrophic response. In the present study, Rb_2 enhanced both the expression of EGF and of the EGFR, which suggests that EGF and/or EGFR transmit the cell proliferation signal from Rb_2. However, another possibility is not excluded that Rb_2 may activate the general metabolism, thus enhancing metabolic activities with concomitant increases of EGF and EGFR level.

Human keratinocytes express several receptors of the integrin family. The enhanced expression of FnR was also observed in wound healing and cellular activation (Ra *et al.*, 1994). In this study, fibroblasts seeded in the collagen matrix of the raft culture expressed fibronectin, and keratinocytes in the basal layer of the raft culture expressed FnR, and both of these events occurred in an Rb_2 concentration dependent manner. This suggests that Rb_2 enhanced cell migration by stimulating both fibroblasts and keratinocytes to produce Fn and FnR, respectively.

A keratin pair represents the sensitive marker for the epidermis state (Nelson and Sun, 1983). Embryonic basal cells during epidermal development and proliferating keratinocytes in culture express keratin 5 and keratin 14, which are considered to be markers of proliferation (Nelson and Sun, 1983). In the present study, the expression of keratins 5 and 14 were proportional to the Rb_2 concentration, which further supports the present result that Rb_2 enhances epidermal cell proliferation in a dose dependent manner.

Collagenase is required for epidermis formation, wound healing, cell detachment from collagen, and keratinocyte migration, the later of which stimulates other cells to produce collagen (Winte, 1962). In the process of wound healing, two hours after wounding the level of collagenase expression peaks (Porras-Reyes *et al.*, 1991), and the conversion of collagenase to its activated form is promoted by EGF. In this study, Rb_2 was found to induce the production of active collagenase I and procollagenase. The active form was present at higher level than the

procollagenase, indicating the possibility of increased keratinocyte migration levels. It is possible that Rb_2 induced expression of EGF and cell migration related factors, thereby leading to induction and activation of collagenase.

Although there have been discrepant observations on cell proliferative activity of Rb_2, this study indicates Rb_2 accelerates cell proliferation, expression of proliferation related factors, and epidermis formation, which in part explains the wound healing effect of Rb_2.

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