

Differential expression of heparin-binding EGF-like growth factor (HB-EGF) mRNA in normal human keratinocytes induced by a variety of natural and synthetic retinoids

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Abstract: It was recently revealed that epidermal growth following topical treatment with all-*trans* retinoic acid (atRA) was at least partly induced by heparin-binding epidermal growth factor-like growth factor (HB-EGF) released from suprabasal keratinocytes. Since proliferation of keratinocytes appears to be one of the critical roles of atRA in depigmentation treatment and promotion of wound healing, HB-EGF is considered suitable for assessing the therapeutic value of topical retinoids. In this study, HB-EGF mRNA expression in normal human keratinocytes after atRA treatment was examined, and the effects of a variety of natural and synthetic retinoids were compared. The results of reverse transcription polymerase chain reaction (RT-PCR) suggested that induction of differentiation increased HB-EGF mRNA expression in cultured keratinocytes. Real-time PCR analyses revealed that HB-EGF mRNA expression was elevated dose-dependently with atRA, peaking at 12 h. This elevation was more prominent in confluent keratinocytes than in subconfluent cells, suggesting that differentiated keratinocytes are more subject to stimulation of HB-EGF expression by atRA than proliferating keratinocytes. HB-EGF mRNA was upregulated in differentiation-induced keratinocytes by all retinoids used in this study at 1 µmol/l, and marked upregulation was seen when treated with three isotypes of retinoic acid (atRA, and 9-*cis* and 13-*cis* retinoic acid). RAR α -selective agonists (Am80, Am580, ER-38925, and TAC-101) and a panagonist of RARs (Re80) caused relatively low elevation of HB-EGF transcripts, as did all-*trans* retinol (Rol) and all-*trans* retinal (Ral). Although another panagonist (Ch55) showed the highest elevation of HB-EGF mRNA, it was relatively cytotoxic at the concentration employed. Ral and Rol were found to upregulate HB-EGF when used at 100 µmol/l to 1 mmol/l, to a similar extent of atRA at 1–10 µmol/l. The capacity of retinoids to upregulate HB-EGF may be an important index for investigation and development of an ideal synthetic retinoid, which has maximum benefits and minimum side-effects

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Introduction

Since the 1970s, topical tretinoin (all-*trans* retinoic acid; atRA) has been widely used for several skin diseases such as acne vulgaris and photoaged skin, with remarkable success. Retinoids have a variety of biological effects on the epidermis and dermis including skin appendices (such as hair follicles

and sebaceous glands), which are mediated by specific nuclear receptors, the RARs (retinoic acid receptors) and RXRs (retinoid X receptors) (1,2). Epidermal hyperplasia is one of the most prominent histological changes in skin seen after treatment with atRA (1,3). This phenomenon has been commonly observed not only *in vivo* in several mammals, but also in skin equivalents using normal

human keratinocytes (NHK) and fibroblasts (data not shown). However, since NHK proliferation was not consistently observed in monolayer-cultured NHK (2,4), the mechanism of keratinocyte proliferation induced by retinoids remained unknown for a long time.

Recently, a study using cultured keratinocytes, organ culture and skin biopsies revealed that transcripts of heparin-binding epidermal growth factor-like growth factor (HB-EGF), a member of the EGF family of growth factors, are induced by treatment with retinoids, suggesting that epidermal hyperplasia after atRA treatment may be mediated, at least in part, by keratinocyte-derived HB-EGF (5). HB-EGF has been shown to be upregulated in actual wound healing far more than other growth factors that accelerate epidermal growth (6). Since then, a paracrine action of HB-EGF released from suprabasal keratinocytes has been found to be a key mechanism of epidermal growth following atRA treatment, in a study using transgenic mice (7). Thus, it is suggested that atRA accelerates keratinocyte differentiation directly, and promotes keratinocyte proliferation indirectly.

Combination topical therapies with atRA and hydroquinone for pigmented skin lesions have been successfully performed since Kligman and Willis proposed their depigmenting formula in 1975 (8). The authors modified the protocol and demonstrated the depigmenting potential of atRA (9,10). Since atRA appeared not to have a suppressive effect on melanogenesis, keratinocyte proliferation and acceleration of epidermal turnover (keratinocyte differentiation) appear to be the two critical effects of atRA in the depigmenting therapies (11). Since the former is mediated by a paracrine action of HB-EGF released from suprabasal keratinocytes, HB-EGF mRNA is thought to be suitable for assessing the therapeutic value of topical retinoids in treating hyperpigmentation or promoting wound healing.

The purposes of this study are to reconfirm the effect of atRA in promoting the expression of HB-EGF mRNA in normal human keratinocytes, and to compare the HB-EGF-promoting abilities of a variety of natural and synthetic retinoids. The capacity of retinoids to upregulate HB-EGF may be an important index for investigating and developing an ideal synthetic retinoid, which has maximum beneficial effects and minimum side-effects.

Methods

Cell isolation and cell culture

Human epidermal keratinocytes isolated from biopsies of healthy skin obtained from young Japanese patients during plastic surgery were used in this study. Keratinocytes were isolated using a

modification of the method reported previously (12). Briefly, the specimens were washed three times in phosphate buffered saline (PBS) and finely shredded with scissors, and incubated with 0.25% trypsin and 0.02% EDTA in PBS for 16–24 h at 4°C. The epithelium was separated from the dermis with forceps, and keratinocytes were isolated from the subepithelial side. Keratinocytes were grown in a modified serum-free keratinocyte growth medium (KGM; Kyokuto Seiyaku, Tokyo), which consists of MCDB153 with high concentrations of amino acids, transferrin (final concentration 10 µg/ml), insulin (5 µg/ml), hydrocortisone (0.5 µg/ml), phosphorylethanolamine (14.1 µg/ml), and bovine pituitary extract (40 µg/ml). The final concentration of Ca²⁺ in the medium was 0.03 mmol/l.

Keratinocytes in a subconfluent state (30% confluency) or those in confluent state (cultured for 48 h after they showed 100% confluency) were used in this study, representing growing and differentiated keratinocytes, respectively.

Reagents

The influence of various kinds of retinoids on HB-EGF mRNA expression of keratinocytes was compared in this study. Five natural retinoids and six synthetic retinoids were used (Fig. 1).

The natural retinoids were tretinoin (all-*trans* retinoic acid; atRA), 13-*cis* retinoic acid (13cRA), 9-*cis* retinoic acid (9cRA), all-*trans* retinol (Rol), and all-*trans* retinal (Ral), all purchased from Sigma (St Louis, MO, USA). 9cRA is known to be a ligand for RXRs and also binds to RARs. The synthetic retinoids were Am80 (4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-carbamoyl] benzoic acid), Am580 (4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido] benzoic acid), Ch55 ((E)-4-[3-(3,5-di-*tert*-butylphenyl)-3-oxo-1-propenyl] benzoic acid), Re80 (4-[1-hydroxy-3-oxo-3-(5,6,7,8-tetrahydro-3-hydroxy-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid), TAC101 (4-[3,5-bis(trimethylsilyl) benzamide] benzoic acid) and ER-38925 (4-[5-(4,7-dimethylbenzofuran-2-yl)pyrrol-2-yl] benzoic acid). All six retinoids were generous gifts from Dr Kagechika (University of Tokyo, Tokyo) (Am80, Am580, Ch55 and Re80), Taiho Pharmaceuticals Co, Ltd (Tokyo, Japan) (TAC101) and Eisai Co, Ltd. (Tokyo, Japan) (ER-38925). Am80, Am580, TAC-101, and ER-38925 are RAR α -selective agonists (13–16), while Ch55 and Re80 are panagonists for all three RAR subtypes (12,17).

All reagents were dissolved in ethanol at 1 mmol/l as stock solutions (for atRA, Rol, and Ral; other stock solutions were also prepared), and 10 µl of each stock solution was added to 10 ml of the culture medium to get the designated final concentrations (for comparison of all retinoids; 1 µmol/l). As a control, 10 µl of ethanol alone was added to 10 ml of the culture medium.

RNA isolation

After removing the culture media and washing twice with PBS, total RNA was obtained with RNeasy[®] Mini Kit (Qiagen, Hilden, Germany). In order to eliminate residual genomic DNA, a RNase-Free DNase Set (Qiagen) was then used. The concentration of each RNA sample was measured with a spectrophotometer (V-530 UV/VIS; Jasco, Tokyo, Japan).

Reverse transcription-polymerase chain reaction analysis

The amount of isolated total RNA was spectrophotometrically measured. A reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using RNA PCR Kit (AMV, Ver. 2.1; TaKaRa, Tokyo, Japan) according to the manufacturer's instructions; 5 µg of total RNA in 100 µl/l of reaction mixture (final concentrations: 5 mmol/l MgCl₂, 1 mmol/l dNTP mixture, 1 U/µl RNase inhibitor, 0.125 µmol/l oligo dT-Adaptor primer, 10 mmol/l Tris-HCl, 50 mmol/l KCl, pH 8.3) containing 25 U of AMV

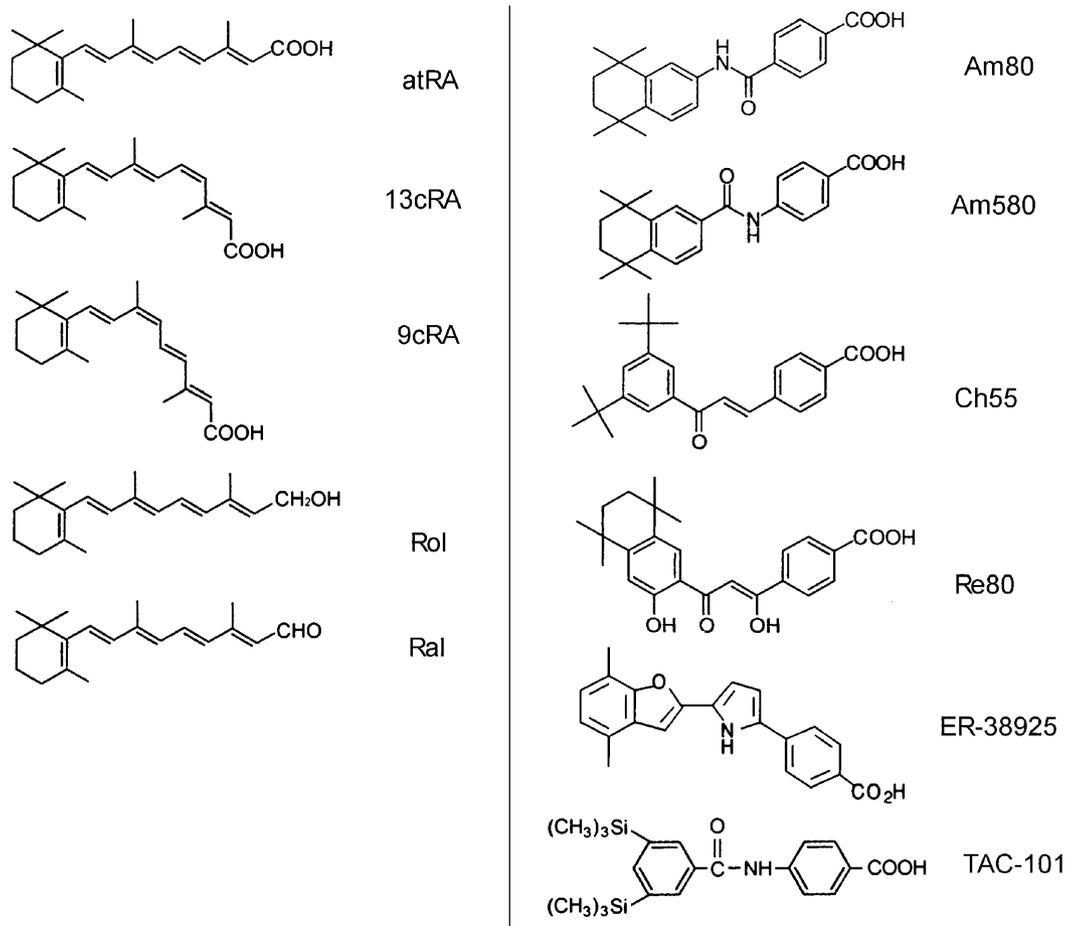


Figure 1. Chemical structures of retinoids used in this study. Left panel: five natural retinoids. atRA: all-trans retinoic acid, 13cRA: 13-cis retinoic acid, 9cRA: 9-cis retinoic acid, Rol: all-trans retinol, Ral: all-trans retinal. Right panel: six synthetic retinoids.

Reverse Transcriptase XL at 42°C for 30 min, followed by inactivation of the enzyme at 99°C for 5 min with Program Temp Control System (PC-700; Astec, Fukuoka, Japan). The control reaction was performed simultaneously under identical conditions, but without reverse transcriptase.

For PCR amplification, 0.5 µl of each cDNA reaction mixture was added to 49.5 µl PCR mixture, containing 5 µl MgCl₂ (25 mmol/l), 5 µl 10× PCR buffer, 1 µl deoxynucleotide mixture (10 mmol/l), 0.5 µl each of the 3' and 5' primers (50 µmol/l each), 0.25 µl *Taq* polymerase, and 37.25 µl DEPC-treated water. Reaction mixtures were amplified using a thermal cycler (Microplate Gradient PC-960G; Corbett Research, Australia). The PCR cycle conditions were: melting for 30 s at 94°C, annealing for 30 s at 59°C, and extension for 1.5 min at 72°C. The oligonucleotide primers used for RT-PCR were as follows; human HB-EGF (forward) 5'-CACACCAAACAAGGAGGAGC-3' and (reverse) 5'-CATGAGAAGCCCCACGATGA-3' (PCR-product size 279 bp); human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward) 5'-GAAGGTGAAGGTCCGAGTC-3' and (reverse) 5'-GAAGATGGTATGGGATTTTC-3' (PCR-product size 226 bp). All RT-PCR products were separated on 2% agarose gels, visualized by ultraviolet B using ethidium bromide staining.

Real time RT-PCR analysis

Expression of HB-EGF transcripts by keratinocytes was quantitatively measured using a real-time quantitative PCR

system (Sequence Detection System ABI PRISM 7700; PE Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed on 96-well optical reaction plates (ABI PRISM™; PE Biosystems). All PCR reaction mixtures contained per well 25 µl of *TaqMan* SYBR[®] Green PCR Master Mix (PE Biosystems), 0.25 µl of forward primer (10 pmol/µl), 0.25 µl of reverse primer (10 pmol/µl), 4 µl of each diluted sample (RT products), and 20.5 µl of distilled water. PCR amplification of each sample was performed with both specific primer pairs of target human HB-EGF gene and human GAPDH gene on the same reaction plate. The oligonucleotide primers used for real-time PCR were as follows: human HB-EGF (forward) 5'-CAGATCTGGACCTTTGAGAGTCA-3' and (reverse) 5'-TCCCGTGCTCCTCCTTGTT-3' (PCR-product size 78 bp); human GAPDH (forward) 5'-GAAGGTGAAGGTCCGAGTC-3' and (reverse) 5'-GAAGATGGT-GATGGGATTTTC-3' (PCR-product size 226 bp). The PCR reaction comprised 40 cycles; denaturing at 95°C (15 s), annealing/extension at 60°C (1 min). For comparison with the HB-EGF mRNA expression, the value of HB-EGF of each sample was normalized by deducting the value of GAPDH from the same sample. In order to eliminate the possibility of contamination of genomic DNA during extraction of total RNA, a control reaction with each primer pair was performed at the same time under identical conditions without reverse transcription, and no amplification was detected. The normalized data were collected from at least four separate experiments.

Statistical analysis

In each experiment, the values of normalized HB-EGF mRNA expression of each sample were divided by the value of the control to obtain data for analysis. Statistical analysis was performed using Student's *t*-test. A value of *P* < 0.05 was considered significant.

Results

RT-PCR analysis

In preliminary experiments, HB-EGF mRNA expression was examined by RT-PCR. HB-EGF mRNA expression was elevated relative to controls in keratinocytes that had been induced to differentiate by incubation for 48 h with high concentrations (1.8 mmol/l) of Ca²⁺ or 2% serum, or both (Fig. 2). This finding suggested that normal human keratinocytes increase HB-EGF mRNA expression when they differentiate.

The investigation of sequential changes of HB-EGF mRNA expression by atRA (1 μM) revealed that HB-EGF mRNA expression peaked at 12 h after atRA administration (Fig. 3). In addition, our results also showed that HB-EGF transcripts were dose-dependently elevated by incubation with atRA for 15 h in the concentration range of 0.01–1 μmol/l (Fig. 4).

HB-EGF mRNA elevation by atRA in subconfluent or confluent keratinocytes

The sequential changes of HB-EGF mRNA elevation by atRA (1 μmol/l) was quantitatively measured with real-time PCR using normal human keratinocytes in subconfluent (30% confluence) and confluent (100% confluence) states (Fig. 5). For confluence, keratinocytes cultured for 48 h after they were first observed to be confluent in culture dishes were used, representing differentiated keratinocytes, as a model of suprabasal keratinocytes. For subconfluence, keratinocytes showing 30% confluency were used immediately,

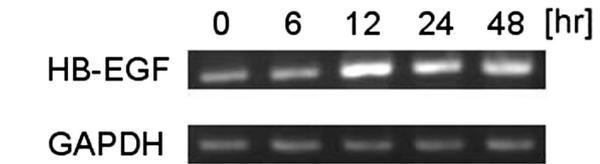


Figure 3. HB-EGF mRNA expression in keratinocytes cultured with medium containing 1 μmol/l atRA for 0, 6, 12, 24, and 48 h. The expression was amplified by RT-PCR.

representing proliferating keratinocytes, as a model of basal keratinocytes. The normalized HB-EGF mRNA level was highest at 12 h, and thereafter decreased in both cases. Throughout the time investigated (0–48 h), the average value of HB-EGF mRNA level was higher in confluent than in subconfluent keratinocytes. Statistical significance between the subconfluent and confluent states was found only at 12 h.

Comparison of HB-EGF mRNA upregulation by natural and synthetic retinoids

The HB-EGF mRNA levels were quantitatively measured with real-time PCR in normal human confluent-state keratinocytes cultured for 15 h with 11 retinoids (0.01 μmol/l, 0.1 μmol/l, and 1 μmol/l atRA; 1 μmol/l for the other retinoids). The results demonstrated that all 11 retinoids studied showed significant upregulation of HB-EGF mRNA, but to different extents (Fig. 6). Ch55, a panagonist of RARs, demonstrated a much higher degree of upregulation of HB-EGF transcripts than the three retinoic acids, but the amount of total RNA isolated from keratinocytes treated with Ch55 was very small compared with that from the other samples, suggesting that Ch55 was cytotoxic to keratinocytes in the concentration employed here (1 μmol/l). Three isotypes of retinoic acid (atRA, 9cRA, and 13cRA) showed higher degrees of upregulation of HB-EGF transcripts than any other natural or synthetic

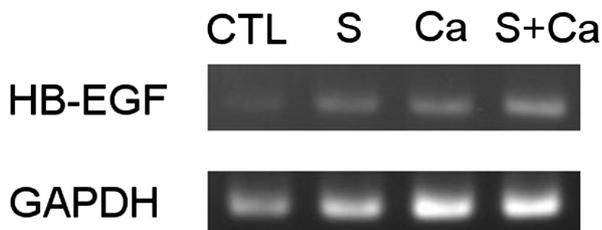


Figure 2. HB-EGF mRNA expression in keratinocytes differentiation-induced by 48 h incubation with medium containing 2% serum or/and 1.8 mmol/l Ca²⁺. It was amplified by RT-PCR and is shown here with the control incubated with normal medium. CTL: control, S: medium with 2% serum, Ca: medium with 1.8 mmol/l Ca²⁺, S + Ca; medium with both 2% serum and 1.8 mmol/l Ca²⁺.

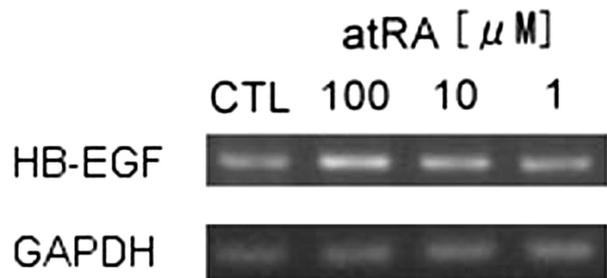


Figure 4. HB-EGF mRNA expression in keratinocytes cultured with medium containing three concentrations (1 μmol/l, 0.1 μmol/l, and 0.01 μM) of atRA for 15 h. The expression was amplified by RT-PCR.

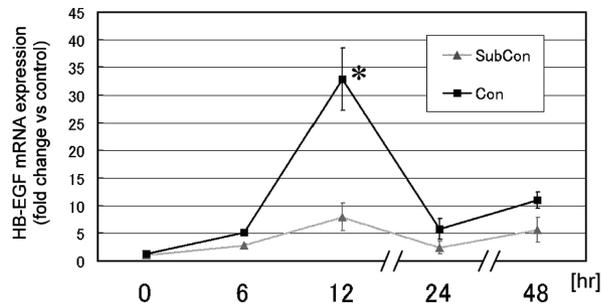


Figure 5. HB-EGF mRNA expression in keratinocytes in subconfluent (30%) or confluent (100%) states cultured with medium containing 1 $\mu\text{mol/l}$ atRA for 0, 6, 12, 24, and 48 h. HB-EGF mRNA was measured quantitatively with real-time PCR system and the normalized value was calculated by deducting the GAPDH value of each sample. The values are demonstrated as relative values to the value obtained from the subconfluent keratinocytes at 0 h, and indicated as mean \pm standard error. * $P < 0.05$.

retinoids except Ch55, although there was no apparent difference among the three retinoic acids. RAR α -selective synthetic agonists (Am80, Am580, ER-38925, and TAC-101) and another panagonist of RARs (Re80) showed a lesser degree of upregulation of HB-EGF transcripts than the three retinoic acids, as did the other two natural retinoids, Rol and Ral.

Comparison of upregulations of HB-EGF mRNA by atRA, Rol and Ral

HB-EGF transcript expression in normal human confluent keratinocytes treated for 15 h with several concentrations of atRA, Rol and Ral were quantitatively measured using real-time PCR. Compared at 1 $\mu\text{mol/l}$ and 10 $\mu\text{mol/l}$, HB-EGF

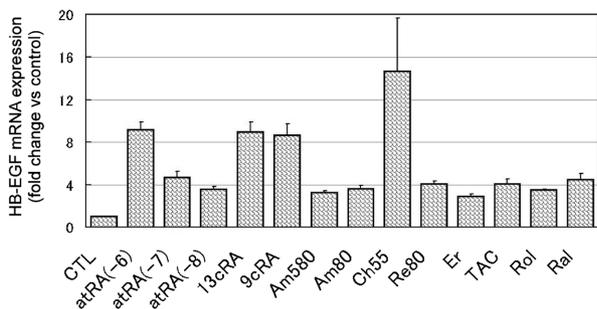


Figure 6. HB-EGF mRNA expression in normal human confluent keratinocytes cultured for 15 h with 1 $\mu\text{mol/l}$ of each retinoid (0.01, 0.1, 1 $\mu\text{mol/l}$ for atRA). HB-EGF mRNA was measured quantitatively with real-time PCR system. The values are demonstrated as relative values to the value obtained from the control in each measurement, and indicated as mean \pm standard error. CTL: control, atRA (-6): 1 $\mu\text{mol/l}$ atRA, atRA (-7): 0.1 $\mu\text{mol/l}$ atRA, atRA (-8): 0.01 μM atRA, Er: ER-38925, TAC: TAC-101. The results of all retinoids are significantly higher than that of control.

was significantly more upregulated by atRA than by Rol or Ral (Fig. 7). When used at concentrations of 100 $\mu\text{mol/l}$ to 1 mmol/l, atRA was cytotoxic, whereas Rol and Ral markedly upregulated HB-EGF to a similar degree as atRA at 1–10 $\mu\text{mol/l}$.

Discussion

It was recently revealed that HB-EGF mRNA can be induced by treatment of retinoids in human keratinocytes and organ-cultured skin, suggesting that epidermal hyperplasia after atRA treatment may be mediated, at least in part, by keratinocyte-derived HB-EGF (5). Thereafter, a paracrine action of HB-EGF released from suprabasal keratinocytes was suggested to be a key mechanism of epidermal growth following atRA treatment in the study, in which dominant-negative RAR α was overexpressed in suprabasal layers of mice, and the response of skin to atRA was examined (7). The present study confirmed that HB-EGF mRNA is markedly induced by atRA in human normal keratinocytes, and suggests that even cell differentiation of keratinocytes alone might increase keratinocyte-derived HB-EGF without retinoid treatment. Although the previous study (5) did not detect a significant difference in HB-EGF upregulation between confluent and subconfluent keratinocytes at 6, 24 or 48 h, our results revealed that differentiated confluent keratinocytes upregulate HB-EGF significantly more than growing subconfluent keratinocytes after 12 h of atRA treatment, supporting the hypothesis of

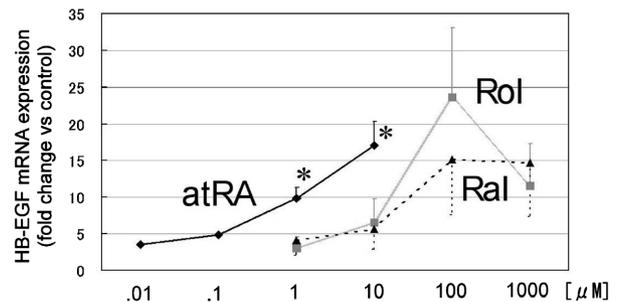


Figure 7. HB-EGF mRNA expression in normal human keratinocytes in confluent condition cultured for 15 h with one of the three natural retinoids (atRA, Rol, and Ral). HB-EGF mRNA was measured quantitatively with real-time PCR system. The values are demonstrated as relative values to the value obtained from the control (normal medium) in each measurement. Four concentrations (0.01 $\mu\text{mol/l}$, 0.1 $\mu\text{mol/l}$, 1 $\mu\text{mol/l}$, and 10 $\mu\text{mol/l}$ for atRA; 1 $\mu\text{mol/l}$, 10 $\mu\text{mol/l}$, 100 $\mu\text{mol/l}$, and 1 mmol/l for Rol and Ral) were employed for each measurement. The values are indicated as mean \pm standard error. At the concentration of 1 $\mu\text{mol/l}$ and 10 $\mu\text{mol/l}$, HB-EGF mRNA expression was significantly higher in keratinocytes treated with atRA than those treated with Rol or Ral. * $P < 0.05$.

Xiao et al. (7) that HB-EGF released from differentiated suprabasal keratinocytes stimulates proliferation of basal keratinocytes. Whether the induction of HB-EGF mRNA is due to ligand-dependent transcription activation of the *HB-EGF* gene or not is to be explored, because the presence of retinoic acid response elements (RARE) has not yet been identified in the promoter region of the *HB-EGF* gene (5,7).

As far as depigmenting treatment is concerned, promotion of keratinocyte proliferation and acceleration of keratinocyte differentiation appeared to be the two main roles of atRA (11). Assuming that HB-EGF released from suprabasal keratinocytes is the main reason for epidermal growth following retinoid treatment *in vivo*, the promotive ability of retinoids on HB-EGF expression can be used as an index for evaluating individual retinoids and developing the synthetic retinoids to promote epidermal growth. However, a reliable index for indicating the ability of retinoids to differentiate keratinocytes has not been found.

All the natural and synthetic retinoids employed in this study significantly upregulated HB-EGF, suggesting that epidermal growth can be induced by a wide range of topical retinoids. However, the extent of HB-EGF upregulation was distinct among those agents. All four RAR α -specific synthetic retinoids, Am80, Am580, ER-38925, and TAC-101, showed relatively low promotion of HB-EGF mRNA compared with all three isotypes of retinoic acid, whereas they demonstrated a higher affinity to RAR α and higher activity in other actions such as growth suppression of neoplasm than did atRA (13–16). The reason for the variation in promotion of HB-EGF mRNA among retinoids remains unknown, but it may be partly due to differential binding affinity to RAR γ . Ninety percent of the RARs expressed in epidermis are RAR γ , and the others are RAR α , while RXR α is a major RXR in skin (2,18). Both RARs and RXRs were reported to have higher expression in the spinous and granular layers compared with the basal layer in normal skin (19). Since RAR- β - or RAR- γ -specific ligands were not evaluated in this study, the relationship between retinoid specificity to subtypes of RARs and HB-EGF expression requires further investigation.

In addition to mediating RARE- or retinoid X receptor responsive elements-dependent transactivation, retinoid receptors can also affect gene expression by inhibiting the action of other transactivation factors, including AP-1 (20). Furthermore, there are some RAR/RXR-independent pathways, such as activation of the mannose-6-phosphate (M6P)/insulin-like growth factor-II (IGF-II) (21).

Human keratinocytes are known to transform Rol into Ral, and then into atRA by two enzymatic steps involving dehydrogenases, and the conversion rate of Rol into atRA depends on the state of keratinocyte differentiation; differentiated keratinocytes can convert at a higher rate than non-differentiated keratinocytes (22). The binding affinity of Rol or Ral to retinoid nuclear receptors is quite low (23), so that their biological activity should result from their oxidative transformation into retinoic acid by epidermal keratinocytes. However, there have been some reports suggesting the existence of other pathways than that mediated by nuclear receptors (24).

Rol is constitutively present in human plasma at 1–2 $\mu\text{mol/l}$ (25) and the upper limit of extracellular Rol concentration in epidermis is thought to be 0.7 $\mu\text{mol/l}$ (26), whereas atRA is present at 4–14 nmol/l in human plasma (27,28). The present results revealed that atRA, Rol and Ral all markedly induce HB-EGF mRNA at pharmacological concentrations, which are 100–1000-fold higher than physiological concentrations. Rol and Ral were in fact able to induce high levels of HB-EGF mRNA when used at much higher (40–100 fold) concentrations than atRA. This means that Rol and Ral, when used at high concentrations, could promote epidermal growth *in vivo* like atRA, although Rol and Ral have been reported to show far fewer side-effects than atRA when used at similar concentrations. Rol and Ral are thought to be more tolerable because of less extensive side-effects, and may be of value in clinical use.

The results indicated the potential for use of Rol or Ral at high concentrations *in vivo*, especially in depigmenting treatments, which need promotion of epidermal growth and turnover. Indeed, our preliminary clinical study with 5–10% Rol aqueous gel suggested similar depigmenting effects to 0.1% atRA aqueous gel (29). Thus, promotion of HB-EGF expression may be used as a promising index for evaluating the depigmenting ability of topical retinoids.

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