Retinoid-Induced Epidermal Hyperplasia Is Mediated by Epidermal Growth Factor Receptor Activation Via Specific Induction of its Ligands Heparin-Binding EGF and Amphiregulin in Human Skin *In Vivo*

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Retinoids are widely used in the treatment of photoaging to stimulate dermal repair. However, retinoids also induce epidermal hyperplasia, which can lead to excessive scaling. Scaling is the major deterrent to topical retinoid therapy. Keratinocyte growth is strongly stimulated via ligand activation of EGFR. We examined regulation of EGFR ligands by retinoids and the role of EGFR in retinoid-induced hyperplasia in human skin *in vivo*. Topical treatment of human skin with all-*trans* retinoic acid (tRA) induces EGFR ligands heparin-binding (HB)-EGF and amphiregulin (AR), and reduces betacellulin mRNA levels. Laser capture microdissection-coupled real-time reverse transcription-PCR reveals that tRA increases HB-EGF mRNA throughout the epidermis, whereas AR induction is limited to basal keratinocytes. Topical tRA activates extracellular signal-regulated kinase 1/2 (Erk1/2) downstream EGFR effectors in human skin *in vivo*. tRA increases the soluble forms of AR and HB-EGF proteins, and induces epidermal hyplasia, in human skin organ culture. Neutralization of HB-EGF or AR with specific antibodies strongly reduces tRA-induced epidermal hyperplasia. Finally, inhibition of EGFR activation by genistein reduces epidermal hyperplasia caused by topical retinoid treatment. These data demonstrate the central role of EGFR activation in retinoid-induced epidermal hyperplasia, and suggest that EGFR inhibitors can mitigate retinoid-induced scaling.

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INTRODUCTION

Retinoids have been widely used for the treatment of numerous dermatological disorders, including psoriasis, acne, skin cancer, and skin aging (Futoryan and Gilchrest, 1994; Zouboulis, 2001). In the treatment of aging, retinoids improve dermal functions, that is, increase fibroblast proliferation (Varani *et al.*, 2000) and collagen production (Griffiths *et al.*, 1993), and decrease matrix metalloproteinase-mediated extracellular matrix degradation (Fisher *et al.*, 1996). However, retinoid-induced improvement of dermis is accompanied by epidermal hyperplasia, which can lead to excessive scaling (Kang *et al.*, 1995). Scaling is the major deterrent to topical retinoid use. The mechanism(s) by which retinoids increase epidermal proliferation in human skin *in vivo* is largely unknown.

All-*trans* retinoic acid (tRA) increases proliferation of basal keratinocytes, inducing accelerated turnover of epidermal cells and thickening of epidermis (Fisher and Voorhees, 1998; Weiss *et al.*, 1988; Varani *et al.*, 2001). The EGFR pathway is the major mitogenic pathway for human keratinocytes (Stoll *et al.*, 2001). The EGFR (ErbB) family contains four members: EGFR (ErbB1), ErbB2, ErbB3, and ErbB4. EGFR, ErbB2, and ErbB3 are expressed in normal human epidermis (Hudson and McCawley, 1998; Stoll *et al.*, 2001). Ligand activation of EGFR directly stimulates keratinocyte proliferation (Hudson and McCawley, 1998), whereas ErbB2 has no known ligand, and ligand activation of ErbB3 is not mitogenic for human keratinocytes (Stoll *et al.*, 2001). EGFR ligands include EGF, amphiregulin (AR), betacellulin (BTC), epiregulin (EPI), heparin-binding EGF-like growth factor (HB-EGF), and transforming growth factor-α (TGF-α) (Marmor *et al.*, 2004). AR and TGF-α have been shown to directly stimulate keratinocyte growth (Coffey *et al.*, 1987).

Ligand activation of EGFR induces receptor homo- or heterodimerization (primarily with ErbB2), which increases EGFR tyrosine phosphorylation. Phosphotyrosine residues on the C-terminus of the receptor act as docking sites for assembly of signaling complexes that mediate cellular responses.

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Abbreviations: AR, amphiregulin; BTC, betacellulin; EPI, epiregulin; Erk, extracellular signal-regulated kinase; HB, heparin binding; HB-EGF, heparin-binding EGF-like growth factor; RAR, RA receptor; RXR, retinoid X receptor; TGF, transforming growth factor-α; tRA, all-trans retinoic acid; tROL, all-trans retinal

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It was previously shown, in a transgenic mouse model, that tRA induces HB-EGF in suprabasal keratinocytes (Xiao *et al.*, 1999). Induction of HB-EGF mRNA has also been reported following tRA treatment of cultured human keratinocytes and human skin (Stoll and Elder, 1998), human skin organ cultures (Varani *et al.*, 2001), and *in vitro* reconstructed skin (Bernard *et al.*, 2002). The use of HB-EGF-specific antibodies or EGFR tyrosine kinase inhibitor reduced tRA-induced epidermal hyperplasia in skin organ culture (Varani *et al.*, 2001).

The aim of this study was to investigate the role of EGFR and its ligands in retinoid-induced epidermal hyperplasia in human skin *in vivo*. Our data support the concept that tRA-induced keratinocyte proliferation is mediated by the activation of EGFR pathway by increased levels of AR and HB-EGF in human skin *in vivo*.

RESULTS

Quantitative gene expression of EGFR ligands in normal human skin *in vivo*

To study EGFR signaling pathway in human epidermis, we assessed gene expression levels of EGFR ligands, AR, BTC, EGF, EPI, HB-EGF, and TGF-α. We quantified relative mRNA levels for each ligand using real-time reverse transcription (RT)-PCR, in skin samples from eight healthy individuals. TGF-α mRNA was the most highly expressed (Figure 1). AR, BTC, EPI, and HB-EGF were expressed at similar levels, approximately five times lower than TGF-α. EGF mRNA levels were 350 times lower than those of TGF-α (Figure 1).

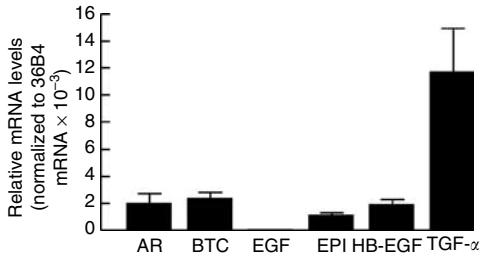

Figure 1. EGFR ligands are expressed in normal human skin *in vivo*. Total RNA was isolated from whole punch biopsies and analyzed by real-time RT-PCR for AR, BTC, EGF, EPI, HB-EGF, and TGF-α mRNA expression in normal human skin, as described in Materials and Methods. Data are mean ± SEM from eight subjects.

Retinoids enhance AR and HB-EGF mRNA levels in human skin *in vivo*

We next treated human skin topically with vehicle, tRA, or all-*trans* retinal (tROL), and determined EGFR ligand mRNA levels. As shown in Figure 2a, 0.1% tRA treatment induced a substantial increase in AR and HB-EGF mRNA. Maximal induction (8.8-fold) of AR mRNA was detected 16 hours after tRA application. Maximal induction (10.6-fold) of HB-EGF mRNA occurred 24 hours after tRA application. AR and HB-EGF mRNA levels remained elevated for at least 4 days of treatment. tRA modestly and transiently increased EPI mRNA levels, which were increased 2.7-fold compared to vehicle-treated skin, 24 hours after initiation of treatment (Figure 2a). EPI mRNA levels were back to control levels 48 hours after tRA application.

tRA induced a marked reduction of BTC mRNA, which was decreased 80% compared to control, 24 hours after tRA application. This inhibition was maintained for at least 4 days of treatment (Figure 2b). No alteration of TGF-α or EGF mRNA levels was detected during the course of tRA treatment of human skin *in vivo* (Figure 2b).

The pattern of EGFR ligand mRNA induction after topical tROL (0.4%) treatment was very similar to that observed with tRA treatment. This observation is consistent with the fact that tROL needs to be converted into tRA in order to exert its activity (Kurlandsky *et al.*, 1994). tROL induced AR and HB-EGF mRNA levels 5.2- and 5.9-fold, respectively, 24 hours after initiation of treatment (Figure 3a). After 4 days

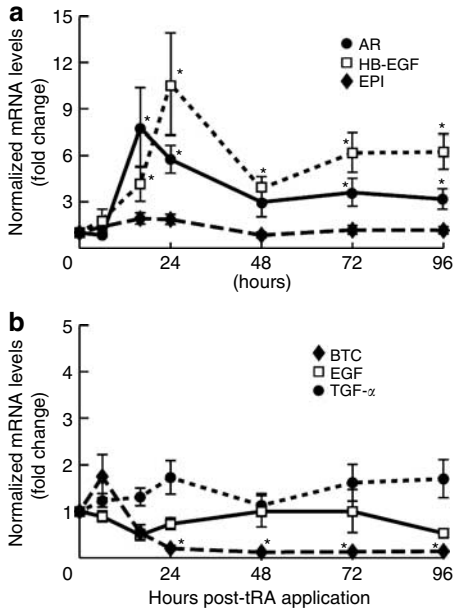

Figure 2. Topical tRA induces AR, HB-EGF, and EPI, decreases BTC, and does not alter EGF and TGF-α in normal human skin *in vivo*. Adult volunteers were treated with vehicle or 0.1% tRA under occlusion as described in Materials and Methods. Biopsies were taken 8, 16, 24, 48, 72, and 96 hours after the beginning of the treatment, and mRNA expression for (**a**) AR, HB-EGF, EPI and (**b**) BTC, EGF, and TGF-α was analyzed by real-time RT-PCR. Data are fold changes in mRNA levels in tRA *versus* vehicle-treated site. Values represent mean ± SEM from eight subjects. *$P<0.05$ *vs* vehicle.

of treatment, AR and HB-EGF mRNA levels remained 5.3- and 8.1-fold higher than controls, respectively. tROL did not alter EPI (Figure 3a), EGF, or TGF-α (Figure 3b) mRNA expression. tROL reduced BTC mRNA synthesis by 80% relative to vehicle-treated skin (Figure 3b). This decrease was maintained for at least 4 days of treatment.

tRA enhances AR and HB-EGF mRNA synthesis in basal and suprabasal keratinocytes in human skin *in vivo*

Using laser capture microdissection, we determined AR, HB-EGF, and BTC mRNA levels in basal and suprabasal keratinocytes in normal human skin. The origin of the cells

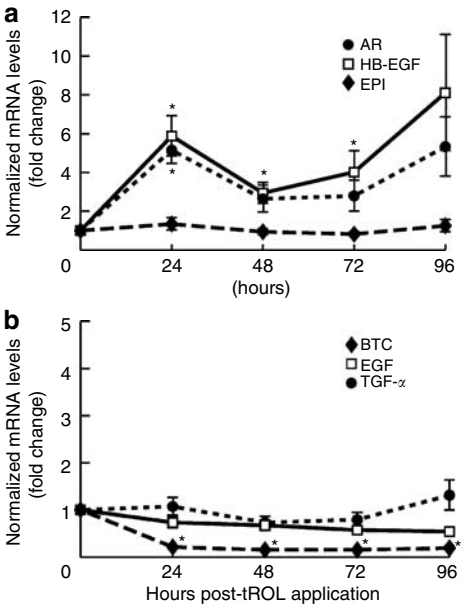

Figure 3. Topical tROL induces AR, and HB-EGF, decreases BTC, and does not alter EGF, EPI, and TGF-α in normal human skin *in vivo*. Adult volunteers were treated with vehicle or 0.4% tROL under occlusion as described in Materials and Methods. Biopsies were taken 24, 48, 72, and 96 hours after the beginning of the treatment, and mRNA expression for (**a**) AR, HB-EGF, EPI and (**b**) BTC, EGF, and TGF-α was analyzed by real-time RT-PCR. Data are fold changes in mRNA levels in tROL *versus* vehicle-treated site. Values represent mean ± SEM from seven subjects. *$P<0.05$ *vs* vehicle.

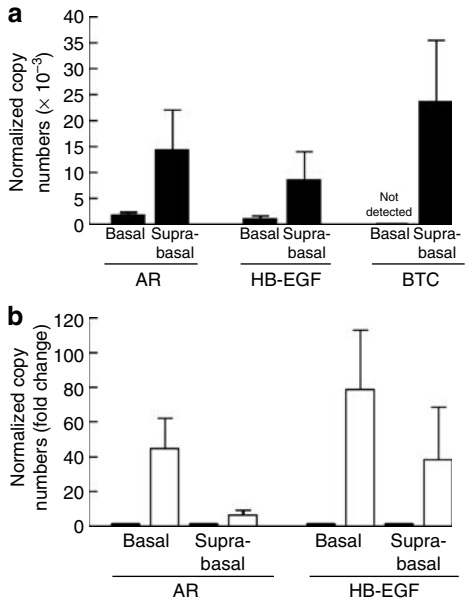

Figure 4. Subepidermal localization of AR, HB-EGF, and BTC mRNA in normal human skin *in vivo* before and after tRA treatment. Adult volunteers were treated for 24 hours with vehicle or 0.1% tRA. Basal and suprabasal keratinocytes were isolated by laser capture microdissection, and RNA from captured cells analyzed by real-time RT-PCR for AR, HB-EGF, and BTC. (**a**) mRNA sublocalization in vehicle-treated skin. (**b**) Fold change in AR and HB-EGF mRNA levels in tRA- (white bars) *versus* vehicle-treated skin (black bars). Data are means ± SEM from four subjects. *$P<0.05$ *vs* (**a**) basal keratinocytes and (**b**) vehicle.

was confirmed by analysis of keratin 5 (basal keratinocytes) and keratin 10 (suprabasal keratinocytes) expression, which is not altered by topical retinoid treatment in human skin *in vivo* (Fisher and Voorhees, 1996). For each sample, keratin 5 mRNA was at least five-fold more abundant in basal relative to suprabasal samples, and keratin 10 mRNA was at least five times higher in suprabasal *vs* basal samples (data not shown). Dermal cells, isolated as control, contained no detectable keratin 5 or 10 mRNA (data not shown).

Analysis of vehicle-treated skin revealed that AR, BTC, and HB-EGF mRNA were predominantly expressed in suprabasal compared to basal keratinocytes (Figure 4a). AR mRNA was detected both in basal and suprabasal cells, with 8.6-fold higher levels in suprabasal *vs* basal keratinocytes. HB-EGF mRNA was near the limit of detection in basal cells (nine-fold lower than in suprabasal cells) and BTC mRNA was not detected in basal keratinocytes.

Twenty-four hours after topical tRA application, AR was induced primarily in the basal layer, whereas HB-EGF was induced in both basal and suprabasal layers (Figure 4b). BTC mRNA could not be reliably measured in either basal or suprabasal layers following tRA treatment (data not shown).

tRA increases AR protein levels in human skin *in vivo*

We next treated human skin topically with vehicle or tRA for 24 hours and prepared epidermal extracts to measure AR and HB-EGF protein by ELISA. AR protein was increased by 3.3-fold after tRA treatment (14.40 ± 10.80 pg/mg total protein in vehicle site *vs* 47.46 ± 15.94 pg/mg total protein in tRA site, $n=3$, $P<0.05$). We were unable to reliability extract and measure HB-EGF protein levels in these samples.

tRA increases the processed forms of AR and HB-EGF proteins in human skin organ cultures

To further investigate tRA regulation of AR and HB-EGF protein expression, we used human skin organ cultures, in which tRA also induces epidermal hyperplasia (Varani *et al.*, 2001). Fresh biopsies were cultured for 3 days with or without 3 μM tRA. At the end of the incubation period, conditioned supernatants were harvested and concentrations of AR and HB-EGF were measured by ELISA. As shown in Figure 5a and b, tRA treatment increased the soluble, secreted AR and HB-EGF proteins by 3.5- and 2.5 fold, respectively.

tRA activates Erk1/2 in human skin *in vivo*

A single 6-mm punch biopsy does not provide sufficient material to detect EGFR phosphorylation by Western blot (Fisher *et al.*, 1998). Therefore, we used extracellular signal-regulated kinase (Erk) phosphorylation as a surrogate for EGFR activation. Ligand activation of EGFR results in increased EGFR tyrosine phosphorylation and activation of downstream effectors such as Erk1/2 (Marmor *et al.*, 2004). Healthy volunteers were treated in buttock skin with vehicle or tRA for 32 hours. Punch biopsies were obtained, and total and phospho-Erk were determined by Western blot. As

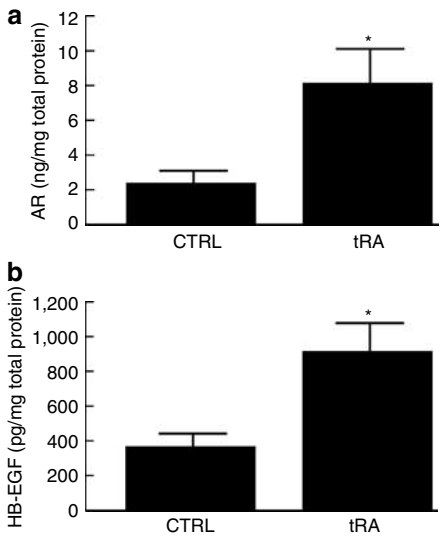

Figure 5. tRA induces AR and HB-EGF proteins in human skin organ culture *in vitro*. Full-thickness punch biopsies were cultured with or without 3 μM tRA for 3 days. At the end of the incubation period, supernatants were harvested and assayed for (**a**) AR and (**b**) HB-EGF proteins by ELISA. Data are mean ± SEM from four experiments. *$P<0.05$ *vs* control.

shown in Figure 6, 32-hour treatment with tRA induced a 2.5-fold increase in Erk1/2 phosphorylation compared to vehicle.

tRA-induced epidermal hyperplasia is blocked by AR and HB-EGF neutralizing antibodies in human skin organ culture

To determine whether AR or HB-EGF were directly involved in tRA-induced epidermal hyperplasia, human skin organ cultures were treated with tRA in the presence of control or neutralizing antibody. tRA induced a marked increase in epidermal thickness (Figure 7). Although control IgGs did not alter tRA-induced epidermal hyperplasia, treatment with either anti-HB-EGF or anti-AR resulted in a marked decrease of RA-induced epidermal hyperplasia, by 51 and 81%, respectively (Figure 7).

Topical genistein inhibits tRA-induced keratinocyte growth, without affecting AR and HB-EGF gene expression levels, in human skin *in vivo*

To further examine the role of EGFR pathway in tRA-induced keratinocyte hyperproliferation in human skin *in vivo*, we utilized the EGFR tyrosine kinase inhibitor genistein, an isoflavone found in soybean (Akiyama *et al.*, 1987; Kang *et al.*, 2003). Subjects were treated topically with vehicle, tROL, or tRA, with or without genistein for 3 days. Figure 8 shows the relative changes in the number of Ki-67-positive cells for each condition. As described previously (Varani *et al.*, 2001), tROL and tRA increased the number of Ki-67-positive cells. Topical treatment of human skin with 1% genistein reduced tROL- and tRA-induced keratinocyte proliferation by approximately 70 and 60%, respectively (Figure 8). Genistein treatment did not alter tROL- and tRA-mediated increase of AR and HB-EGF mRNA levels (data not shown).

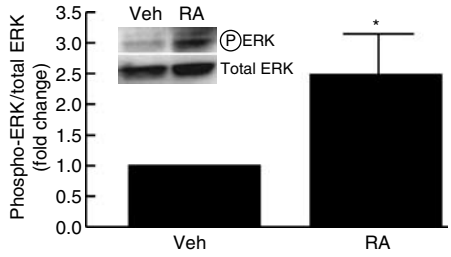

Figure 6. tRA enhances Erk1/2 phosphorylation in human skin *in vivo*. Adult volunteers were treated with vehicle or 0.1% tRA under occlusion for 32 hours. Epidermal lysates were prepared and analyzed for phospho- and total Erk1/2 by Western blot. Band intensities were quantified by PhosphorImager. Data are mean ± SEM from four experiments. Inset shows a representative Western blot. *$P<0.05$ *vs* vehicle.

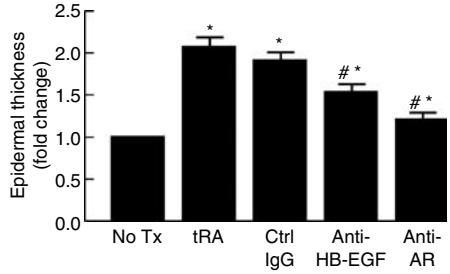

Figure 7. Retinoid-induced epidermal hyperplasia is blocked by AR and HB-EGF neutralizing antibodies. Full-thickness punch biopsies were cultured with or without 3 μM tRA, supplemented with blocking antibodies to HB-EGF, AR, or control IgGs. At the end of the incubation period, epidermal thickness was measured after hematoxylin and eosin staining. Data are mean ± SEM from nine experiments. *$P<0.05$ *vs* control; #$P<0.05$ *vs* tRA.

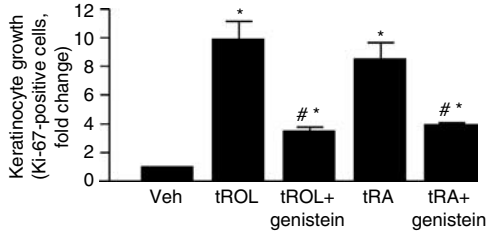

Figure 8. Topical genistein inhibits tRA-induced epidermal hyperproliferation in human skin *in vivo*. Adult volunteers were treated for 3 days with vehicle, 0.4% tROL, or 0.1% tRA with or without 1% genistein. Biopsies were taken at the end of the treatment period and keratinocyte growth was determined as Ki-67-positive cells. Data are mean ± SEM from five subjects. *$P<0.05$ *vs* vehicle; #$P<0.05$ *vs* retinoid alone.

DISCUSSION

Taken together, recent studies conducted in cell culture, organ culture, and in animal models support the concept that retinoid-induced keratinocyte proliferation is mediated by EGFR activation through induction of its ligand HB-EGF (Stoll and Elder, 1998; Xiao *et al.*, 1999; Varani *et al.*, 2001). We have extended these observations to human epidermis *in vivo*. Our results indicate that, in contrast to what is observed in mouse skin, tRA-mediated modulation of EGFR ligands is

not limited to HB-EGF. In human skin *in vivo*, retinoids induce AR and HB-EGF and reduce BTC mRNA steady-state levels, without affecting basal levels of EPI, TGF-α, or EGF. We find that EGFR activation plays a central role in retinoid-induced epidermal cell growth in human skin *in vivo*. Blocking EGFR tyrosine kinase activity by genistein reduces retinoid-induced epidermal cell growth in human skin *in vivo*.

We noted several differences in the tRA regulation of EGFR ligands between human and mouse epidermis. First, HB-EGF mRNA induction reaches a maximal level ~6 hours after tRA application in mouse skin (Xiao *et al*., 1999), whereas induction is maximal 24 hours after initiation of treatment in human skin (Figure 2a). This difference in kinetics likely reflects that the stratum corneum, which provides a barrier for penetration of tRA into the living layers of skin, is approximately twice as thick in human skin compared to mouse skin (~17 *vs* ~9 μm) (Marks, 2004). Another striking difference between mouse and human skin is that tRA induces AR in human, but not in mouse skin (Xiao *et al*., 1999). Interestingly, AR is increased after tRA treatment in human skin organ culture (Figure 5a), but not in reconstructed skin *in vitro* (Bernard *et al*., 2002). In mouse skin, AR mRNA is detectable by Northern blot, but is unchanged after topical tRA treatment (Xiao *et al*., 1999). AR mRNA levels are inducible in mouse skin in response to other conditions that disrupt the skin permeability barrier, for example, by solvents or tape stripping (Liou *et al*., 1997).

The mechanism by which retinoids induce EGFR ligand synthesis remains unclear. Retinoids exert their biological effects through binding to nuclear RA receptors (RARs), and retinoid X receptors (RXR), which belong to the superfamily of nuclear hormone receptors. Among the different isoforms, adult human skin expresses predominantly RAR-γ and RXR-α (Elder *et al*., 1991; Fisher *et al*., 1994). Upon binding to their ligand, retinoid receptors heterodimerize and act as transcription factors, which bind to specific regions of promoter sequences called RA response elements. Transgenic mouse studies revealed that targeted overexpression of dominant-negative RXR-α (Feng *et al*., 1997), or RAR-γ (Xiao *et al*., 1999), or targeted disruption of RXR-α and RAR-γ (Chapellier *et al*., 2002) in suprabasal keratinocytes, prevents topically applied tRA from stimulating proliferation of epidermal keratinocytes. Taken together, these studies indicate that suprabasal expression of functional RAR/RXR is required for tRA-mediated epidermal hyperplasia and HB-EGF induction, at least in mice. However, examination of the published sequences of HB-EGF (Fen *et al*., 1993) and AR (Plowman *et al*., 1990) does not reveal any putative RA response elements. Although the existence of yet unidentified RA response elements upstream or downstream of the previously reported promoter regions is difficult to rule out, a direct modulation of HB-EGF or AR promoters seems unlikely. Alternatively, RAR/RXR may regulate other transcription factor(s) that would directly regulate AR and HB-EGF gene expression. The identity of such transcription factor(s) remains to be determined.

Interestingly, tRA downregulates BTC in human skin. BTC is also reduced in psoriasis lesions, characterized by hyperproliferative epidermis (Piepkorn *et al*., 2003). The membrane-bound precursor of BTC contains an RGD sequence, which binds extracellular matrix protein receptors (integrins), in its extracellular domain. It has been suggested that BTC acts as a membrane-anchored integrin ligand, mediating cell–cell interactions (Dunbar and Goddard, 2000). In this case, downregulation of BTC would decrease cell–cell cohesion, which conceivably could promote cell proliferation and/or migration. Further experiments are required to test this possibility.

Topical tRA treatment induced a marked increase in AR protein levels in whole epidermal extracts. However, we were unable to reproducibly quantify HB-EGF protein because their levels were very low, approximately 10-fold lower than AR, despite a similar mRNA copy number in human skin. Whether this difference reflects a lower extraction yield and/or a shorter half-life of the protein is unknown. However, it is interesting to note that, in human keratinocytes in culture, activation of EGFR pathway shortens the half-life of HB-EGF transcripts and stabilizes AR mRNA (Stoll and Elder, 1999). Further experiments would be needed to determine whether the two transcripts are similarly regulated in human epidermis *in vivo*.

Using human skin organ cultures, we consistently measured increased levels of both AR and HB-EGF proteins after tRA treatment. Our results demonstrate that (i) tRA increases AR and HB-EGF protein levels, and (ii) the two growth factors are processed and released in their soluble forms after retinoid treatment.

Our data demonstrate that AR, BTC, and HB-EGF mRNA are expressed primarily in the suprabasal cells of normal epidermis. Predominantly suprabasal expression of AR was previously demonstrated by *in situ* hybridization in human skin (Schelfhout *et al*., 2002). tRA treatment induces HB-EGF in both basal and suprabasal layers, whereas AR induction is more limited to basal keratinocytes. These findings reveal that these two growth factors are regulated differently in a manner that is dependent on the stage of keratinocyte maturation. The mechanisms involved in this maturation-dependent regulation remain to be elucidated.

Basal keratinocytes are the proliferative cells of the epidermis. To stimulate basal keratinocyte proliferation, AR and HB-EGF must act in a paracrine/autocrine and juxtacrine manner. We were unable to determine whether AR and HB-EGF made by basal and suprabasal layers are equally functional. However, we demonstrate that AR and HB-EGF both contribute to and are direct mediators of retinoid-induced epidermal hyperplasia, using blocking antibodies in skin organ cultures. Control IgGs, at higher concentrations, caused nonspecific inhibition of keratinocytes growth. Therefore, we were not able to reliably assess the combined effect of AR and HB-EGF antibodies.

We demonstrated that a 32-hour tRA treatment induces activation of the EGFR pathway in human epidermis *in vivo* as measured by phosphorylated Erk1/2. To further demonstrate the central role of EGFR activation in tRA-mediated

epidermal hyperplasia, we used genistein. Genistein is a potent inhibitor of EGFR tyrosine kinase activity (Akiyama *et al.*, 1987) that has previously been shown to block UV-induced EGFR activation in human skin *in vivo* (Kang *et al.*, 2003), and to reduce retinoid-mediated epidermal hyperplasia in skin organ culture (Varani *et al.*, 2004). Here, we demonstrate that topical genistein significantly reduces retinoid-mediated basal keratinocyte proliferation and epidermal hyperplasia. Interestingly, we observe that genistein does not prevent retinoid-mediated upregulation of AR or HB-EGF, demonstrating that EGFR activation is downstream of AR and HB-EGF induction. In contrast, it has been shown that the EGFR/Erk pathway regulates the transcription of several EGFR ligands in cultured keratinocytes. For instance, activation of EGFR by EGF increases the transcription of AR, HB-EGF, and TGF-α (Stoll and Elder, 1999), and the selective MAPK/Erk kinase inhibitor PD98059 downregulates HB-EGF mRNA (Pascall *et al.*, 2000).

The most prominent side effect of topical retinoid treatment is excessive scaling (Weiss *et al.*, 1988). Scaling is a direct consequence of retinoid-induced hyperplasia, as keratinocytes that are induced to proliferate in the lower epidermis must mature into corneocytes, and desquamate at the skin surface. Our study demonstrates that tRA-induced epidermal hyperplasia is mediated by EGFR ligands AR and HB-EGF, in both basal and suprabasal epidermal layers. Inhibition of EGFR activity by compounds such as genistein may mitigate unwanted scaling caused by topical retinoid treatment, and thereby improve compliance.

MATERIALS AND METHODS

Retinoid treatment and tissue procurement

All procedures involving human subjects were approved by the University of Michigan Institutional Review Board, and all subjects provided written informed consent before being enrolled in the study. Experiments were carried out in adherence to the Declaration of Helsinki Principles.

Adult volunteers were treated topically with tRA, tROL, or vehicle (seven parts 95% ethanol and three parts propylene glycol (vol/vol)), under occlusion, on sun-protected buttock skin. Duration of treatments varied from 8 to 96 hours as indicated in figure legends. For each subject, treated sites were separated by at least 2.5 cm. Vehicle treatment was for 24 hours or 4 days, and did not alter EGFR ligand transcript levels, compared to untreated skin (data not shown). In some experiments, 1% genistein was applied to skin simultaneously with tRA, tROL, or vehicle. After treatment, full-thickness punch biopsies were obtained from each site. For RNA and protein isolation, tissue samples (6-mm punches) were snap-frozen in liquid nitrogen and stored at −80°C until processing. For immunohistochemistry and laser capture microdissection studies, tissue samples (4-mm punches) were embedded in Tissue-Tek OCT compound (Miles, Naperville, IL) before freezing.

RNA isolation and quantitative real-time reverse transcription-PCR

Total RNA from vehicle- or retinoid-treated human skin was extracted using a commercial kit (RNeasy, Qiagen, Chatsworth, CA). The levels of mRNA expression were quantified by real-time PCR as described previously (Quan *et al.*, 2001). PCR primers and probes, produced by the custom oligonucleotide synthesis service (Applied Biosystems, Foster City, CA), are described in Table 1. Target gene mRNA levels were normalized to 36B4 (housekeeping gene) mRNA levels. Efficiency of real-time RT-PCR reactions was determined using cDNA standards for TGF-α, HB-EGF, and AR.

Laser capture microdissection

Frozen sections (7–10 μm thick) were collected onto polyethylene naphthalate foil-coated slides (Leica Microsystems, Bannockburn, IL) and allowed to air-dry for 5 minutes. Sections were fixed in 75% ethanol for 30 seconds and stained by immersion with hematoxylin and eosin, using the following protocol: 30 seconds DEPC-treated water, 15 seconds hematoxylin, 30 seconds DEPC-treated water, 10 seconds eosin, 30 seconds 75% ethanol, 30 seconds 95% ethanol, and twice 30 seconds 100% ethanol. Slides were allowed to air-dry for 5 minutes, and laser capture microdissection was performed using a Leica AS LMD to obtain basal (first cell layer in contact with the basement membrane) and suprabasal (remaining epidermis) keratinocytes. Captured cells were collected directly into RNA

Table 1. Sequences of human primers and probes used for real-time PCR

Gene (labeling)	Sense primer (5′→3′)	Antisense primer (5′→3′)	Probe (5′→3′)
AR (FAM)	GGCCATTATGCTGCTGGAT	TGTGGTCCCCAGAAAATGGT	TGTGGTCCCCAGAAAATGGT
BTC (FAM)	GGGAGATGCCGCTTCGT	TGCTCCAATGTAGCCTTCATCA	CCGAGCAGACGCCCTCCTGTG
EGF (FAM)	AATACCGTTAAGATACAGTGTAGGCACTTTA	ATCACAACTCATTTTGGCAAAATC	CTCCTCATTGGCGTGGTCCATGCTGAT
Epiregulin (FAM)	GGCTCCTTCATCGAATGCTAA	CAGGATAAACGTAGAGGAAGAACAGA	CCTTTGAGTAGAGTCTCCCTGGATCACATACCA
HB-EGF (FAM)	TGGCCCTCCACTCCTCATC	GGGTCACAGAACCATCCTAGCT	CACCCACCTTTGCCACA
TGF-α (FAM)	AGGACAGCACTGCCAGAGATG	CGACGGAGTTCTTGACAGAGTTT	CTGTGCAGCCTTTTGTGGGCCTTC
Keratin 5 (FAM)	CCAGGAGAGCCCCATTCC	GAAACCTGAAGGCTGATTTGAAG	TGGTCTCCCGTGCCGCAGTTC
Keratin 10 (FAM)	TCTGGCCGCGGATGACT	CCATTGATGTCGGCTTCCA	CTCATGCGCAGGTTCAACTCTGTCTCAT
36B4 (VIC)	ATGCAGCAGATCCGCATGT	TTGCGCATCATGGTGTTCTT	CGCGGGAAGGCTGTGGTGCT

AR=amphiregulin; BTC=betacellulin; HB-EGF=heparin-binding EGF-like growth factor; PCR=polymerase chain reaction; TGF-α=transforming growth factor-α.

isolation lysis buffer (RNeasy kit; Qiagen), supplemented with RNase inhibitor (Applied Biosystems) before RNA extraction.

Human skin organ cultures

Two-mm full-thickness punch biopsies of sun-protected buttock skin were used for skin organ cultures, conducted as described previously (Varani *et al.*, 2001). For some experiments, culture medium was supplemented with 3 μRA, 10 μg/ml neutralizing antibodies to HB-EGF (R&D Systems, Minneapolis, MN), 25 μg/ml neutralizing antibodies to AR (R&D Systems), or the corresponding concentration of control IgG (R&D Systems). At the end of the incubation period, supernatants were harvested and assayed for HB-EGF and AR protein (see below) and tissue was fixed in 10% formalin for histological examination after hematoxylin and eosin staining. Epidermal thickness was measured as described previously (Varani *et al.*, 2001).

Epidermal extract preparation and Western blotting

Epidermal extracts were prepared from frozen punch biopsies, after removing the dermis using a sterile scalpel. Epidermis was homogenized in ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.6, 1% Ipegal, 0.25% Na deoxycholate, 150 mM NaCl, 1 mM EDTA, Protease inhibitors (complete mini; Roche, Indianapolis, IN), 1 mM NO_3VO_4, 1 mM NaF), and vortexed in the presence of glass beads (Biospec, Bartlesville, OK). After 10 minutes centrifugation at 10,000 *g* and 4°C, supernatant was assayed for total protein using BioRad Protein Assay reagent (BioRad, Hercules, CA), and bovine serum albumin as a standard. Western blotting were performed on 60 mg of epidermal extract, under denaturing conditions, according to the method described previously (Quan *et al.*, 2001). Antiphospho-Erk1/2 (Cell Signalling, Danvers, MA) and anti-total Erk (Transduction Laboratories, BD Biosciences, Franklin Lakes, NJ) were used at 1:1,000 dilutions.

AR and HB-EGF ELISA

AR ELISA was performed using a DuoSet human amphiregulin ELISA kit (R&D Systems), according to the manufacturer's instructions. HB-EGF ELISA was performed according to the method described by Yamada *et al.* (1998) and modified by Vinante *et al.* (1999). Amounts of AR and HB-EGF protein were normalized with the total amount of protein.

Immunohistochemisty for Ki-67

Seven-micrometer frozen sections were fixed in paraformaldehyde and methanol, and stained with an antibody to the proliferation marker Ki-67 (MAB4190; Chemicon, Temecula, CA) used at 1:500 dilution. Presence of tissue-bond primary antibodies was visualized using a secondary antibody –peroxidase and AEC staining kit (Sigma, St Louis, MO). The number of positive cells per unit length of epidermis was evaluated using ImagePro Plus software.

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). Comparisons among groups were made with the paired Student's *t*-test. When necessary, logarithmic transformations of the data were made to achieve normalcy before analysis; however, the figures depict the data on the untransformed scale. All *P*-values are two-tailed, and considered significant when less than 0.05.

CONFLICT OF INTEREST

The authors states no conflict of interest.

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REFERENCES

Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N *et al.* (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* 262:5592–5

Bernard F-X, Pedretti N, Rosdy M, Deguercy A (2002) Comparison of gene expression profiles in human keratinocyte mono-layer cultures, reconstituted epidermis and normal human skin; transcriptional effects of retinoid treatments in reconstituted human epidermis. *Exp Dermatol* 11: 59–74

Chapellier B, Mark M, Messaddeq N, Calléja C, Warot X, Brocard J *et al.* (2002) Physiological and retinoid-induced proliferations of epidermis basal keratinocytes are differently controlled. *EMBO J* 21:3402–13

Coffey RJ, Derynck R, Wilcox JN, Bringman TS, Goustin AS, Moses HL *et al.* (1987) Production and auto-induction of transforming growth factor in human keratinocytes. *Nature* 328:817–20

Dunbar AJ, Goddard C (2000) Structure-function and biological role of betacellulin. *Int J Biochem Cell Biol* 32:805–15

Elder JT, Fisher GJ, Zhang Q-Y, Eisen D, Krust A, Kastner P *et al.* (1991) Retinoic acid receptor gene expression in human skin. *J Invest Dermatol* 96:425–33

Fen Z, Dhadly MS, Yoshizumi M, Hilkert RJ, Quertermous T, Eddy RL *et al.* (1993) Structural organization and chromosomal assignment of the gene encoding the human heparin-binding epidermal growth factor-like growth factor/diptheria toxin receptor. *Biochemistry* 32: 7932–8

Feng X, Peng ZH, Di W, Li XY, Rochette-Egly C, Chambon P *et al.* (1997) Suprabasal expression of a dominant-negative RXR alpha mutant in transgenic mouse epidermis impairs regulation of gene transcription and basal keratinocyte proliferation by RAR-selective retinoids. *Genes Dev* 11:59–71

Fisher GJ, Datta SC, Talwar HS, Wang ZQ, Varani J, Kang S *et al.* (1996) Molecular basis of sun-induced premature skin ageing and retinoid antagonism. *Nature* 379:335–9

Fisher GJ, Talwar HS, Lin J, Lin P, McPhillips F, Wang ZQ *et al.* (1998) Retinoic acid inhibits induction of c-Jun protein by ultraviolet radiation that occurs subsequent to activation of mitogen-activated protein kinase pathways in human skin *in vivo*. *J Clin Invest* 101: 1432–40

Fisher GJ, Talwar HS, Xiao JH, Datta SC, Reddy AP, Gaub M-P *et al.* (1994) Immunological identification and functional quantitation of retinoic acid and retinoid X receptor proteins in human skin. *J Biol Chem* 269: 20629–35

Fisher GJ, Voorhees JJ (1996) Molecular mechanisms of retinoid actions in skin. *FASEB J* 10:1002–13

Fisher GJ, Voorhees JJ (1998) Molecular mechanisms of photoaging and its prevention by retinoic acid: ultraviolet irradiation induces MAP kinase signal transduction cascades that induce AP-1-regulated matrix metalloproteinases that degrade human skin *in vivo*. *J Investig Dermatol Symp Proc* 3:61–8

Futoryan T, Gilchrest BA (1994) Retinoids and the skin. *Nutr Rev* 52:299–310

Griffiths CEM, Russman AN, Majmudar G, Singer RS, Hamilton TA, Voorhees JJ (1993) Restoration of collagen formation in photodamaged human skin by tretinoin (retinoic acid). *N Engl J Med* 329:530–5

Hudson LG, McCawley LJ (1998) Contributions of the epidermal growth factor to keratinocyte mobility. *Microsc Res Technol* 43:444–55

Kang S, Chung JH, Lee JH, Fisher GJ, Wan YS, Duell EA *et al.* (2003) Topical *N*-acetyl cysteine and genistein prevent ultraviolet-light-induced signaling that leads to photoaging in human skin *in vivo*. *J Invest Dermatol* 120:835–41

Kang S, Duell EA, Fisher GJ, Datta SC, Wang ZQ, Reddy AP *et al.* (1995) Application of retinol to human skin *in vivo* induces epidermal hyperplasia and cellular retinoid binding proteins characteristic of retinoic acid but without measurable retinoic acid levels or irritation. *J Invest Dermatol* 105:549–56

Kurlandsky SB, Xiao JH, Duell EA, Voorhees JJ, Fisher GJ (1994) Biological activity of all-*trans* retinol requires metabolic conversion to all-*trans* retinoic acid and is mediated through activation of nuclear retinoid receptors in human keratinocytes. *J Biol Chem* 269: 32821–7

Liou A, Elias PM, Grunfeld C, Feingold KR, Wood LC (1997) Amphiregulin and nerve growth factor expression are regulated by barrier status in murine epidermis. *J Invest Dermatol* 108:73–7

Marks R (2004) The stratum corneum barrier: the final frontier. *J Nutr* 134: 2017S–21S

Marmor MD, Skaria KB, Yarden Y (2004) Signal transduction and oncogenesis by Erb/HER receptors. *Int J Radiat Oncol Biol Phys* 58:903–13

Pascall JC, Ellis PD, Brown KD (2000) Characterization of the rat heparin-binding epidermal growth factor-like growth factor gene promoter. *Biochim Biophys Acta* 1492:434–40

Piepkorn M, Predd H, Underwood RA, Cook PW (2003) Proliferation–differentiation relationships in the expression of heparin-binding epidermal growth factor-related factors and erbB receptors by normal and psoriatic human keratinocytes. *Arch Dermatol Res* 295:93–101

Plowman GD, Green JM, McDonald VL, Neubauer MG, Disteche CM, Todaro GJ *et al.* (1990) The amphiregulin gene encodes a novel epidermal growth factor-related protein with tumor-inhibitory activity. *Mol Cell Biol* 10:1969–81

Quan T, He T, Voorhees JJ, Fisher GJ (2001) Ultraviolet irradiation blocks cellular responses to transforming growth factor-β by down-regulating its type-II receptor and inducing Smad7. *J Biol Chem* 276:26349–56

Schelfhout VRJ, Coene ED, Delaey B, Waeytens AAT, De Rycke L, Deleu M *et al.* (2002) The role of heregulin-α as a motility factor and amphiregulin as a growth factor in wound healing. *J Pathol* 198:523–33

Stoll SW, Elder JT (1998) Retinoid regulation of heparin-binding EGF-like growth factor gene expression in human keratinocytes and skin. *Exp Dermatol* 7:391–7

Stoll SW, Elder JT (1999) Differential regulation of EGF-like growth factor genes in human keratinocytes. *Biochem Biophys Res Commn* 265:214–21

Stoll SW, Kansra S, Peshick S, Fry DW, Leopold WR, Wiesen JF *et al.* (2001) Differential utilization and localization of erbB receptor tyrosine kinases in skin compared to normal and malignant keratinocytes. *Neoplasia* 3: 339–50

Varani J, Kelley EA, Perone P, Lateef H (2004) Retinoid-induced epidermal hyperplasia in human skin organ culture: inhibition with soy extract and soy isoflavones. *Exp Mol Pathol* 77:176–83

Varani J, Warner RL, Gharaee-Kermani M, Phan SH, Kang S, Chung JH *et al.* (2000) Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin. *J Invest Dermatol* 114: 480–486

Varani J, Zeigler M, Dame MK, Kang S, Fisher GJ, Voorhees JJ *et al.* (2001) Heparin-binding epidermal-growth-factor-like growth factor activation of keratinocyte erbB receptors of retinoid therapy. *J Invest Dermatol* 117:1335–41

Vinante F, Marchi M, Rigo A, Scapini P, Pizzolo G, Cassatella MA (1999) Granulocyte–macrophage colony-stimulating factor induces expression of heparin-binding epidermal growth factor-like growth factor/diptheria toxin receptor and sensitivity to diptheria toxin in human neutrophils. *Am Soc Hematol* 94:3169–77

Weiss JS, Ellis CN, Headington JT, Tincoff T, Hamilton TA, Voorhees JJ (1988) Topical tretinoin improves photoaged skin. *JAMA* 259:527–32

Xiao JH, Feng X, Di W, Peng ZH, Li LA, Chambon P *et al.* (1999) Identification of heparin-binding EGF-like growth factor as a target in intercellular regulation of epidermal basal cell growth by suprabasal retinoic acid receptors. *EMBO J* 18:1539–48

Yamada A, Kawata S, Tamura S, Kiso S, Higashiyama S, Umeshita K *et al.* (1998) Plasma heparin-binding EGF-like growth factor levels in patients after partial hepatectomy as determined with an enzyme-linked immunosorbent assay. *Biochem Biophy Res Commun* 246:783–7

Zouboulis CC (2001) Retinoids – which dermatological indications will benefit in the near future? *Skin Pharmacol Appl Skin Physiol* 14:303–15