Human Antigen R-mediated mRNA Stabilization Is Required for Ultraviolet B-induced Autoinduction of Amphiregulin in Keratinocytes*

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Background: Amphiregulin (AREG) is a ligand for the EGF receptor (EGFR) and autoinduces its own expression.

Results: Human antigen R (HuR), an mRNA-binding protein, stabilizes AREG mRNA in ultraviolet B (UVB)-exposed keratinocytes.

Conclusion: HuR is a key regulator for UVB-mediated autoinduction of AREG expression.

Significance: Clarifying the mechanisms underlying the production of EGF family members is crucial for understanding cancers caused by aberrant EGFR signaling.

All members of the EGF family are produced as transmembrane precursors that are proteolytically processed into soluble forms by disintegrin and metalloproteinases (ADAMs) for autocrine/paracrine pathways. In turn, the ligand-activated EGF receptor (EGFR) induces the expression of EGF family members, so-called “autoinduction.” However, it is not well understood how this autoinduction occurs. In this study, we investigated the molecular mechanism of the autoinduction of amphiregulin (AREG), a member of the EGF family. We found that ultraviolet B (UVB) exposure increased the AREG mRNA level by stabilization of its mRNA in a human immortalized keratinocyte cell line, HaCaT. The 3′ UTR of AREG mRNA was responsible for binding to an mRNA-binding protein, human antigen R (HuR), and the interaction between AREG mRNA and HuR was enhanced by UVB. Inducible knockdown of HuR expression significantly decreased AREG mRNA stability. Interestingly, treatment of HaCaT cells with an EGFR inhibitor, an EGFR neutralizing antibody, or an ADAM inhibitor destabilized AREG mRNA. In the case of ADAM inhibition, administration of soluble AREG restored the mRNA level, indicating that the stabilization occurs in a shedding-dependent manner of EGFR ligands. The HuR dependence of AREG mRNA and protein expression was also confirmed in human primary keratinocytes. Taken together, we propose a novel mechanism by which HuR regulates the stability of AREG mRNA in keratinocytes after UVB exposure and suggest that targeting of HuR functions might be crucial for understanding skin cancers caused by aberrant EGF family member-EGFR signaling.

UV irradiation has a wide variety of effects on skin and is the main etiological factor for the induction of nonmelanoma skin cancers, such as basal cell carcinomas and squamous cell carcinomas (1). It is well known that UV irradiation produces reactive oxygen species as a result of photolysis of water molecules and induces DNA damage through the formation of single-strand breaks and oxidation of bases like 7,8-dihydro-8-oxoguanine, which plays a crucial role in carcinogenesis (2). Generation of reactive oxygen species by UV irradiation also activates the EGFR receptor (EGFR)3 and inactivates phosphatases, thereby synergistically enhancing growth factor receptor signaling in keratinocytes (3, 4). Because aberrant activation of the EGFR-ligand system following UV irradiation can lead to skin carcinogenesis, it is essential to understand how such aberrations could be brought about. Of note, EGF family members can induce their own gene expression via autocrine/paracrine activation of the EGFR, which is known as “autoinduction” (5, 6). Therefore, one of the key issues for understanding the aberrant regulation is to clarify the autoinduction mechanism of EGFR ligands because the amounts of EGFR ligands directly affect the activity of the EGFR and subsequent intracellular signaling in keratinocytes.

The ligands of EGFR consist of seven members of the EGF family, namely EGF, amphiregulin (AREG), heparin-binding

3The abbreviations used are: EGF, EGF receptor; HuR, human antigen R; AREG, amphiregulin; HB-EGF, heparin-binding EGF-like growth factor; ADAM, a disintegrin and metalloproteinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; UVB, ultraviolet B; AcD, actinomycin D; qPCR, quantitative PCR; DOX, doxycycline; miR, microRNA; ARE, AU-rich element.
EGF-like growth factor (HB-EGF), transforming growth factor α (TGFα), epiregulin, betacellulin, and epiogen (7, 8). One of the rich sources for the production of EGF family members is epidermal keratinocytes. All EGFR family members are produced as type I transmembrane precursor forms (pro-forms) in keratinocytes (7, 8) and become soluble factors via a regulated proteolytic process termed “ectodomain shedding.” Under steady-state conditions, AREG is prominently expressed in the normal human skin epidermis and cultured keratinocytes (9–11). In addition, the expression of AREG increased in the psoriatic epidermis (12), and transgenic expression of AREG in basal keratinocytes induces psoriasis-like phenotypes such as marked hyperkeratosis and cutaneous inflammation (13). Furthermore, not only AREG but also other EGF family members induce their expression mutually via EGFR activation, so-called “auto- and cross-induction” (5, 6, 14). These observations indicate the importance of an EGFR-ligand system in the growth, differentiation, and migration of keratinocytes in skin.

EGFR activation is basically mediated by direct ligands. However, the EGFR is also transactivated by non-direct ligands, including extracellular stimuli such as UV irradiation, reactive oxygen species, and wounding, or various G protein-coupled receptor ligands and cytokines (7). In the process of EGFR transactivation, ectodomain shedding and binding of direct ligands are crucial events that subsequently lead to the activation of intracellular signaling pathways. Ectodomain shedding of the pro-forms is mainly mediated by a disintegrin and metalloproteinase (ADAM) 17, which is also a type I transmembrane protein (15, 16). A wide variety of stimuli, including UV irradiation (17–19), wounding (20), hypoxia (21), many types of G protein-coupled receptor agonists (22, 23), and 12-O-tetradecanoylphorbol-13-acetate (TPA) (19, 24, 25) activate ADAM17 and other ADAMs and evoke ectodomain shedding of EGF family ligands that subsequently transactivate the EGFR.

We recently identified cell surface annexins as essential regulators of the UVB-induced ectodomain shedding of the AREG pro-form (pro-AREG) (19). Annexin A8 and annexin A9 positively regulate UVB-induced EGFR transactivation via ADAM17-mediated pro-AREG shedding, whereas annexin A2 negatively regulates the process in human primary keratinocytes. A previous report also showed that ectodomain shedding of pro-AREG is required for UV-induced EGFR transactivation in keratinocytes (18).

Although much attention has been focused on ADAM-mediated shedding or EGFR downstream signaling events, the precise molecular mechanism that increases the AREG mRNA and protein levels after UV exposure is poorly understood. In this study, we investigated the stability of AREG mRNA by focusing on its UTR. We found that an mRNA-binding protein, human antigen R (HuR) associated with the 3′ UTR of AREG mRNA in response to UVB exposure, leading to enhanced mRNA stabilization. We also evaluated the significance of the role of EGFR activation through metalloproteinase-mediated ectodomain shedding in UVB-induced AREG mRNA stabilization.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The following antibodies were used in this study: goat polyclonal antibody against the extracellular region of pro-AREG (anti-AREG-N, catalog no. AF262, R&D Systems), rabbit polyclonal anti-GFP antibody (catalog no. NO.598, MBL), mouse monoclonal anti-EGFR (clone 225, Calbiochem) (26), mouse monoclonal anti-HuR antibody (catalog no. sc-5261) and anti-lamin A/C antibody (catalog no. sc-7292, Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-β-actin antibody (clone AC-15) and β-tubulin antibody (clone JDR.3B8, Sigma-Aldrich), and mouse IgG (Chemicon). Actinomycin D (AcD) and AG1478 were purchased from MP Biomedicals and Calbiochem, respectively. KB-R7785 was a gift from Carna Biosciences, Inc. Recombinant human AREG (catalog no. 262-AR) was obtained from R&D Systems.

**Cell Culture**—An immortalized non-transformed keratinocyte cell line, HaCaT, was grown in DMEM containing 10% FBS. Human primary keratinocytes were cultured in optimized nutrient medium, MCDB153 (Nissui), supplemented with 5 μg/ml insulin, 0.5 μM hydrocortisone, 0.1 mM ethanalamine, 0.1 mM phosphoethanolamine, and 150 μg/ml bovine hypothalamic extract as described previously (5). All cells were cultured in a humidified incubator with 5% CO₂ at 37 °C.

**UVB Irradiation**—Cells were exposed to UVB with FL20S30 fluorescent sunlamps (Toshiba Medical Supply). A Kodakel filter was mounted in front of the tubes to filter out any wavelengths below 290 nm. The irradiation intensity was monitored using a photodetector. The day before UVB exposure, the cells were incubated in serum-free medium. 30 min before UVB exposure, the serum-free medium was refreshed. After the indicated period of time post-UV exposure, total RNA or whole cell lysates were prepared. For determination of the mRNA stability, AcD (2 μg/ml) was added immediately after UVB exposure, and the cells were lysed at various time points.

**Quantitative PCR (qPCR)**—Total RNA was isolated using ISOGON II (Nippon Gene), and reverse transcription of RNA (1.5 μg) was performed with a high-capacity RNA-to-cDNA kit (Applied Biosystems) according to the protocol of the manufacturer. After first-strand synthesis, qPCR was performed using a FastStart Universal SYBR Green Master (ROX) mixture (Roche) with a 7300 real-time PCR system (Applied Biosystems). The qPCR primers for the EGFR family ligands and COX-2 were the same as those in previous reports (27, 28). The detection primers for GAPDH and 18S were as follows: 5′-catcag-agatgtagaacaagcct-3′ and 5′-agtcctccacgataccaagatt-3′ for GAPDH and 5′-ccctataacctttgatgtgctg-3′ and 5′-cataatggactctgtaagatt-3′ for 18S.

**Luciferase Reporter Assay**—A plasmid encoding a human AREG promoter-driven luciferase reporter gene (pGL4-AREG promoter) was constructed by inserting the PCR-amplified promoter fragment of AREG (nucleotides −858 to −210) into pGL4.15[<luc2P/Hygro] (Promega). The nucleotide numbers refer to the promoter sequence (29). As an internal control vector, pGL4.74[<hRLuc/TK] (Promega) was used. Human primary keratinocytes were transiently transfected with the luciferase reporter plasmids using Lipofectamine 2000 reagent (Invitrogen). The day before the luciferase reporter assay, the keratinocytes were incubated in bovine hypothalamic extract-free medium. 30 min before UVB or TPA treatment, the culture medium was refreshed. Cells were treated with UVB light (50 ml/cm²) or TPA (100 nM) and then incubated for 8 h. The promoter activities were analyzed using a dual-luciferase assay.
HuR Regulates UVB-induced AREG mRNA Stabilization

A

![Graph: AREG mRNA stability](image)

B

![Graph: AREG promoter activity](image)

C

![Graph: Remaining AREG mRNA](image)

**FIGURE 1.** UVB irradiation increases the stability of AREG mRNA. A, HaCaT cells were exposed to UVB irradiation at the indicated doses and then incubated for 2 h (a and c) or exposed to UVB irradiation (50 mJ/cm²) and then incubated for the indicated times (b and d). The AREG (a and b) and COX-2 (c and d) mRNA levels were quantified by qPCR, normalized by the respective 18S rRNA levels, and expressed as fold changes relative to UVB-untreated cells. Data represent means ± S.E. *, *p < 0.05; **, the human AREG promoter reporter plasmid and an internal control plasmid were transfected into human primary keratinocytes. Cells were treated with UVB irradiation (50 mJ/cm²) or TPA (100 nM) and then incubated for 8 h. AREG promoter activity was analyzed by measuring luciferase activity. The firefly luciferase activities were normalized by the Renilla luciferase activities. Data represent means ± S.E. ***, *p < 0.01. C, HaCaT cells were exposed to UVB irradiation (50 mJ/cm²). AcD (2 μg/ml) was added immediately, and the cells were incubated for the indicated times, followed by analysis of the AREG (a) and COX-2 (b) mRNA levels by qPCR. The AREG and COX-2 mRNA levels were normalized by the respective 18S rRNA levels and expressed as percentages of the level at the 0-min time point. †, the calculated half-life of AREG mRNA was 4.7 ± 1.4 h in UVB (+) cells.

reporter assay system (Promega). The assays were repeated at least three times, and the firefly luciferase (AREG promoter) activities were normalized by the Renilla luciferase (internal control) activities.

Subcellular Fractionation—Cells were fractionated into cytosolic and nuclear fractions using a ProteoExtract subcellular proteome extraction kit (Merck Biosciences) according to the protocol of the manufacturer. Cytosolic fractions (buffer I extracts) and nuclear protein fractions (buffer III extracts) were separated by SDS-PAGE, and the presence of HuR was detected by Western blot analysis. β-tubulin and lamin A/C were used as markers for the specific enrichment of cytosolic and nuclear fractions, respectively.

Western Blot Analysis—Each sample was subjected to SDS-PAGE, and the separated proteins in the gels were transferred to nitrocellulose membranes. The membranes were blocked with 4% skim milk in PBS-T (PBS containing 0.05% Tween 20) for 30 min, followed by incubation with primary antibodies. After washing with PBS-T, the membranes were incubated with appropriate HRP-conjugated secondary antibodies. Immunoreactivity was detected using the ECL Plus Western blotting detection system (GE Healthcare).

Biotin Pull-down Assays—Synthesis of biotinylated transcripts and analysis of HuR bound to biotinylated RNA were performed as described previously (30). Briefly, pGEM-T vectors (Promega) containing the 5′ UTR, coding region, 3′ UTR, or 3′ UTR deletion (970–1166) of AREG mRNA were prepared. These plasmids were used as templates to transcribe biotinylated RNAs with T7 polymerase and biotin RNA labeling mix (Roche). DNase I was added to remove the template DNA. The biotinylated transcripts (1 μg) were incubated with HaCaT cell lysates (100 μg protein) for 1 h at room temperature. Complexes were isolated using paramagnetic streptavidin-conjugated Dynabeads (Invitrogen), and the bound proteins were analyzed by Western blotting.

RNA Immunoprecipitation Assay—Immunoprecipitation was performed with an RNA-binding protein immunoprecipitation kit (Millipore). Briefly, HaCaT cells (1 × 10⁷) were lysed and immunoprecipitated with normal mouse IgG or an anti-HuR antibody (5 μg) at 4 °C overnight. Protein A/G magnetic beads were added to each sample, followed by mixing for 1 h at 4 °C. HuR-bound RNA was isolated with TRIzol reagent (Invitrogen). The cDNA was used as a template for qPCR.

Lentiviral Vector-mediated Gene Knockdown—On the basis of a self-inactivating lentivirus vector system (31), we constructed a single lentiviral vector for doxycycline (DOX)-inducible gene knockdown (32). The system carries two components of the transgene in a single lentiviral vector. One expression unit contains the reverse Tet-controlled transactivator protein (rtTA) (TaKaRa Bio, Inc.) together with a blasticidin-resistance gene through control of the CAG promoter. The other unit comprised a microRNA (miR)-based specific stem-loop sequence designed using a Block-iT miR RNAi system (Invitrogen) together with GFP downstream of the Tet-responsive pro-
moter. The lentiviral vector plasmid DNA, CS-CA-rtTA-
IRBsd-TRE-EmGFP-miR, was utilized for silencing the human
HuR gene expression. Two HuR-specific sequences designed
using Block-iT miR RNAi Select (Invitrogen) (#1, top sequence
of Hmi405122, 5'-H11032
-TGCTGAGAACATGTCTTCTACGTCC-
TGTTTTGGCCACTGACTGACAGGACGTAAGACATGT-
TCT-3'; #2, top sequence of Hmi405123, 5'-H11032
-TGCTG-
CAAACCTGTAGTCTGATCCACGTTTTGGCCACTGAC-
TGACGTGGATCACTACAGGTTTG-3'; H11032
) were selected and
inserted individually between the 5'-miR and 3'-miR flanking
sequences of the plasmid, respectively. As a negative control,
the sequence for silencing luciferase (top sequence, 5'-H11032
-TGCTG-
GTATTCAGCCCATATCGTTTCAGTTTTGGCCACTGA-
CTGACTGAAACGATGGGCTGAATA-3'; H11032
) was used. Effi-
cient inhibition of the luciferase activity by this construct was
confirmed by a transient luciferase assay using a pGL2-based
reporter plasmid (data not shown). Lentiviral particles were
generated by a standard transfection procedure. After trans-
duction of the transgenes, each pool of HaCaT cells resistant to
blasticidin was used in experiments. Knockdown of HuR pro-
deral was performed by treatment with DOX (1 g/ml) for 4
days.

Immunofluorescence Staining—For immunofluorescence
staining, cells were fixed with 4% paraformaldehyde and incub-
bated with the primary antibody against HuR at 4 °C overnight
and then stained with the Cy3-conjugated anti-mouse IgG
(Jackson ImmunoResearch Laboratories, Inc.). Nuclei were
stained with Hoechst33342 (Invitrogen). The fluorescent
images were captured by an IX70 fluorescence microscope
(Olympus).

RNA Interference—Control and HuR (Hs_ELAVL1_12)
siRNAs were purchased from Qiagen. Transfection of each
siRNA (20 nM) was performed with Lipofectamine RNAiMAX
(Invitrogen) according to the protocol of the manufacturer.

Statistical Analysis—All assays were performed independ-
ently three times. The results are represented as means ± S.E.
*p < 0.05. Two groups were compared using Student's t
test. Analysis of variance with Scheffe’s post-hoc test was used for multiple
comparisons. Values of p < 0.05 were considered statistically
significant.

RESULTS
Induction of AREG mRNA Expression by UVB Irradiation—
To analyze the effects of UVB irradiation on AREG mRNA
expression, HaCaT cells were exposed to multiple doses of UVB
irradiation. Then, cells were incubated for 2 h, and total RNAs
were isolated. qPCR analysis revealed that AREG mRNA was
induced in a dose-dependent manner with a 3-fold increase at
the peak dose of 50 mJ/cm² (Fig. 1A, a). At this dose of UVB
irradiation (50 mJ/cm²), AREG mRNA expression significantly
increased in a time-dependent manner up to 6 h of incubation (Fig. 1A, b). Consistent with previous reports (33, 34), UVB irradiation-induced increases in COX-2 mRNA were confirmed as a positive control in HaCaT cells (Fig. 1A, c and d). These data indicate that UVB up-regulates AREG mRNA expression in HaCaT cells.

UVB Increases AREG mRNA Stability—Because the amount of mRNA depends on the balance between the rates of synthesis and degradation, we initially examined whether UVB irradiation affects AREG promoter activity. We performed a luciferase reporter assay with a plasmid containing a 648-bp fragment of the AREG promoter upstream of the luciferase gene. It has been demonstrated that this promoter region responds to several stimuli, such as TPA, hypoxia, parathyroid hormone, and insulin, and is regulated by the Wilms tumor suppressor transcription factor in U2OS osteosarcoma cells (35–38). We transfected human primary keratinocytes with the AREG promoter-luciferase plasmid together with an internal control plasmid. On the following day, the cells were exposed to UVB irradiation (50 mJ/cm²) or TPA (100 nM), and then the luciferase activity was measured after 8 h of incubation. As reported previously (36), TPA treatment induced AREG promoter activity 3-fold in this assay (Fig. 1B). In contrast, we were unable to detect any UVB-dependent promoter activation derived from this reporter plasmid after UVB irradiation (Fig. 1B).

Therefore, we next examined AREG mRNA stability using the transcriptional inhibitor AcD. As shown in Fig. 1C, a, UVB irradiation prolonged the half-life of AREG mRNA to over 2.4 times longer than the control (> 4.0 h versus 1.7 h). As a positive control, we examined the stability of COX-2 mRNA, which also increased in response to UVB irradiation to ~ 2.1 times longer than the control (3.0 h versus 1.4 h) (Fig. 1C, b). Although these data do not exclude the possibility that a UVB irradiation-responsive element exists outside of the cloned AREG promoter region (648 bp), these findings strongly suggest that UVB treatment can stabilize AREG mRNA.

HuR Binds to the 3′ UTR of AREG mRNA—On the basis of analyses of the AREG genomic structure, Plowman et al. (29) identified AU-rich elements (AREs) in the 3′ UTR of AREG mRNA that might control mRNA stability (Fig. 2A). Taken together with our results (Fig. 1), these observations prompted us to examine the possible involvement of mRNA-binding factors in the regulation of AREG mRNA stability or instability. In this study, we focused on one of the most studied ARE-binding proteins, HuR, which is also known as embryonic lethal abnormal vision-like 1 (ELAVL1). HuR is a ubiquitously expressed mRNA-binding protein of the ELAV family and mediates cellular responses to DNA damage and other types of stress through the regulation of cell growth and proliferation-controlling genes (39).

We hypothesized that HuR regulates UVB-induced AREG mRNA stabilization and, therefore, first examined whether HuR binds to AREG mRNA by RNA immunoprecipitation assays. HaCaT cells were exposed to UVB irradiation (50
HuR Regulates UVB-induced AREG mRNA Stabilization

FIGURE 4. HuR is required for UVB-induced AREG mRNA stabilization. A, independent pools of HuR or control miR-expressing HaCaT cells were cultured with or without DOX (1 μg/ml) for 4 days and exposed to UVB irradiation (50 ml/cm²). Acid (2 μg/ml) was added immediately, and the cells were incubated for the indicated times. The AREG mRNA levels were quantified by qPCR, normalized by the respective 18S rRNA levels, and expressed as percentages of the level at the 0-min time point. †, the calculated half-life of AREG mRNA was 5.4 ± 1.4 h in #1 DOX (−), 4.4 ± 0.1 h in control DOX (−), and 4.2 ± 0.5 h in control DOX (+) cells. Data represent means ± S.E. *, p < 0.05. n.s., not significant. B, the COX-2 mRNA levels were quantified and normalized as in A. †, the calculated half-life of COX-2 mRNA was 4.9 ± 2.0 h in #1 DOX (−) and 4.3 ± 1.3 h in #2 DOX (−) cells. Data represent means ± S.E. *, p < 0.05. n.s., not significant.

HuR Regulates the Stability of AREG mRNA after UVB Irradiation—To examine the effects of HuR on the stabilization of endogenous AREG mRNA after UVB irradiation, we knocked down the expression of HuR using a miR-based RNAi system (31, 32). We constructed three lentivirus vectors carrying a DOX-regulatable Emerald GFP (EmGFP) and a miR expression cassette as shown in Fig. 3A, and established the independent pools of HuR-miR-expressing cells (#1 and #2) and control-miR-expressing cells (Control). Immunocytochemical analyses showed that administration of DOX for 4 days effectively achieved knockdown of HuR protein in GFP-positive cells (Fig. 3B). As judged by Western blotting analyses, the knockdown efficiency of HuR at the protein level was ~75% in DOX-treated cells (Fig. 3C). It is assumed that HuR is abun-
dantly localized within the nucleus and that its export to the cytoplasm seems to be a major prerequisite for the stabilizing effects on target ARE-containing mRNAs (40). After UVB irra-

mRNA was also enriched in the anti-HuR antibody precipitate under UVB irradiation (Fig. 2B).

Next, to determine which region of the AREG mRNA is responsible for the interaction with HuR, a pull-down assay was performed using four kinds of in vitro-transcribed AREG mRNAs (Fig. 2A). The AREs were included in a construct of 3′ UTR but not in that of the 5′ UTR, coding region, or 3′ UTR-del. HaCaT cell lysates were incubated with the in vitro-transcribed biotinylated RNAs. The RNA-protein complexes were pulled down by streptavidin beads, and the precipitates were analyzed by Western blotting with the anti-HuR antibody. The HuR protein was specifically coprecipitated with the AREG 3′ UTR but not with the 5′ UTR or coding region (Fig. 2C). Importantly, the amount of coprecipitated HuR protein markedly decreased when AREG 3′ UTR-del was pulled down (Fig. 2C). Taken together with the result shown in Fig. 2B, these findings suggest that, in response to UVB irradiation, HuR associates with the 3′ UTR of AREG mRNA in AREs-dependent manner.

To determine whether the AREG 3′ UTR contributes to mRNA stabilization, we constructed a luciferase reporter containing the whole 3′ UTR (Fig. 2D). When this plasmid was transfected into HaCaT cells, a 5-fold decrease in the luciferase activity was observed (Fig. 2E), suggesting that the 3′ UTR actually induced mRNA instability under steady-state conditions. In contrast to endogenous AREG mRNA, however, UVB treatment did not induce up-regulation of this exogenous reporter activity (Fig. 2E). These findings suggest that the 3′ UTR is necessary for the interaction between AREG mRNA and HuR, although the 3′ UTR alone is not sufficient for the UVB irradiation-induced mRNA stabilization. It is possible that RNA elements in the 5′ UTR and coding region, or their binding factors, cooperate with the AREs in the 3′ UTR and HuR.
When HuR was knocked down, the half-life of AREG mRNA after UVB irradiation was diminished from $t_{1/2} = 4.0$ h to $t_{1/2} = 2.4$ h and from $t_{1/2} = 3.7$ to $t_{1/2} = 2.0$ h in #1 and #2 cells, respectively (Fig. 4A, #1 and #2). In the control cells, DOX administration did not significantly alter the half-life of AREG mRNA (Fig. 4A, Control). We also observed that the half-life of COX-2 mRNA decreased when HuR expression was suppressed in #1 and #2 cells (Fig. 4B, #1 and #2) but not in control cells (Control). These findings suggest that HuR plays a critical role in the stabilization of endogenous AREG mRNA after UVB irradiation in keratinocytes.

UVB-induced AREG mRNA Stabilization Is Mediated through EGFR—On the basis of the results shown in Fig. 4, HuR was indicated to increase the AREG mRNA level in response to UVB exposure. To know whether HuR-mediated AREG mRNA stabilization constitutes a part of the AREG-EGFR autoinduction loop, we analyzed the stability of AREG mRNA under EGFR activity-suppressed conditions. Thirty minutes before UVB irradiation (50 mJ/cm²), HaCaT cells were incubated with or without 1 μM AG1478. AcD (2 μg/ml) was added immediately and the cells were incubated for the indicated times, followed by analysis of the AREG mRNA levels by qPCR. The AREG mRNA levels were normalized by the respective 18S rRNA levels and expressed as percentages of the level at the 0-min time point. Data represent means ± S.E., $p < 0.05$. B, 30 min before UVB irradiation (50 mJ/cm²), HaCaT cells were incubated with or without 10 μM KB-R7785. AcD (2 μg/ml) and recombinant AREG (100 ng/ml) were added immediately, and the AREG mRNA levels were analyzed as in A. †, the calculated half-life was 5.3 ± 1.5 h in KB-R7785 + rhAREG cells. C, HaCaT cells were incubated with mouse IgG or anti-EGFR neutralizing antibody (5 μg/ml) on ice for 30 min and then exposed to UVB irradiation (50 mJ/cm²). AcD (2 μg/ml) was added immediately, and the AREG mRNA levels were analyzed as in A. †, the calculated half-life was 5.0 ± 0.6 h in control IgG cells. Data represent means ± S.E., $p < 0.05$. A, 30 min before UVB irradiation (50 mJ/cm²), HaCaT cells were incubated with or without 1 μM AG1478. AcD (2 μg/ml) was added immediately and the cells were incubated for the indicated times, followed by analysis of the AREG mRNA levels by qPCR. The AREG mRNA levels were normalized by the respective 18S rRNA levels and expressed as fold changes relative to UVB-untreated cells. Data represent means ± S.E., $p < 0.05$.

FIGURE 5. The EGFR signaling pathway is critical for UVB irradiation-induced AREG mRNA stabilization. A, 30 min before UVB irradiation (50 mJ/cm²), HaCaT cells were treated with or without 1 μM AG1478. AcD (2 μg/ml) was added immediately and the cells were incubated for the indicated times, followed by analysis of the AREG mRNA levels by qPCR. The AREG mRNA levels were normalized by the respective 18S rRNA levels and expressed as percentages of the level at the 0-min time point. Data represent means ± S.E., $p < 0.05$. B, 30 min before UVB irradiation (50 mJ/cm²), HaCaT cells were incubated with or without 10 μM KB-R7785. AcD (2 μg/ml) and recombinant AREG (100 ng/ml) were added immediately, and the AREG mRNA levels were analyzed as in A. †, the calculated half-life was 5.3 ± 1.5 h in KB-R7785 + rhAREG cells. C, HaCaT cells were incubated with mouse IgG or anti-EGFR neutralizing antibody (5 μg/ml) on ice for 30 min and then exposed to UVB irradiation (50 mJ/cm²). AcD (2 μg/ml) was added immediately, and the AREG mRNA levels were analyzed as in A. †, the calculated half-life was 5.0 ± 0.6 h in control IgG cells. Data represent means ± S.E., $p < 0.05$.
UVB exposure (50 mJ/cm²), HaCaT cells were treated with an EGFR kinase inhibitor, AG1478, and then total RNAs were isolated at the indicated times. As shown in Fig. 5A, treatment of HaCaT cells with AG1478 diminished UVB irradiation-induced AREG mRNA stability from t1/2 = 3.6 h to t1/2 = 1.3 h. We also examined AREG mRNA stability using a metalloproteinase inhibitor, KB-R7785, because UV irradiation induces ADAM17-mediated ectodomain shedding of EGFR ligand proforms, including pro-AREG, and the release of soluble EGFR ligands, leading to transactivation of EGFR in keratinocytes (18, 19). Treatment with KB-R7785 destabilized AREG mRNA from t1/2 = 3.9 h to t1/2 = 2.0 h, whereas addition of soluble form AREG rescued the stability of AREG mRNA from t1/2 = 2.0 h to t1/2 > 4.0 h (Fig. 5B). Moreover, the addition of the anti-EGFR neutralizing antibody destabilized AREG mRNA from t1/2 > 4.0 h to t1/2 = 3.0 h (Fig. 5C). These findings indicate that the UVB-induced stabilization of AREG mRNA depends on EGFR activation and also suggest that AREG mRNA regulation could constitute a critical part of the AREG-EGFR autoinduction loop, enabling continuous production of AREG in response to UVB exposure.

**DISCUSSION**

After UV exposure, rapid activation of cell survival signaling is essential to protect keratinocytes against UV irradiation-induced reactive oxygen species production and subsequent cell damage. UV irradiation also activates the ADAM family of metalloproteinases, which mediates ectodomain shedding and release of AREG into the extracellular environment (18, 19). Because the cell surface pro-AREG level decreases after ectodomain shedding, quick replenishment of an appropriate amount of pro-AREG is supposed to be necessary for the continuous survival, proliferation, and migration of keratinocytes. In this study, we have revealed a critical role of HuR in the UVB irradiation-induced stabilization of AREG mRNA and have also shown that mRNA stabilization occurred in an EGFR-dependent manner. Considering the rapid replenishment of pro-AREG, we speculate that stabilization of mRNA as well as rapid activation of gene expression could be important parameters for efficient protein production.

Although the AREs in the AREG mRNA had been implicated to act as mRNA-stabilizing consensus motifs (29), it was not demonstrated whether AREs actually affect mRNA stability. In this study, we have provided evidence that the mRNA-stabilizing factor HuR became associated with the 3’ UTR of AREG mRNA in response to UVB exposure and prevented its degradation. Because knockdown of HuR reduced UVB irradiation-induced AREG mRNA stability and pro-AREG protein production in human primary keratinocytes, the regulatory system of mRNA stabilization plays an essential role in the autoinduction of AREG expression, which definitely contributes to signal augmentation in response to UVB exposure.

FIGURE 7. Model of UVB irradiation-induced AREG mRNA stabilization. In response to UVB exposure, activated ADAM17 induces ectodomain shedding of pro-AREG. The released soluble AREG activates EGFR through an autocrine/paracrine mechanism, followed by activation of intracellular signaling that includes HuR activation. It should be noted that HuR-mediated mRNA stabilization could contribute to the production of pro-AREG even without a rapid increase in AREG gene transcription. CR, coding region.
way bypasses this step, enabling a more prompt supply of pro-
AREG on the cell surface. Furthermore, it is well known that
AREG induces the expression of other EGF family members,
HB-EGF, TGFα and epiregulin, by the mechanism known as
cross-induction (6). We speculate that UV irradiation-induced
stabilization of AREG mRNA is one of the novel mechanisms
that ensures rapid amplification and maintenance of EGF fam-
ily ligand-EGFR signaling for the adaptation of keratinocytes
to changing environmental conditions, such as UV irradiation-
induced cell damage.

HuR binds to AREs in the 3’ UTRs of various kinds of
mRNAs including c-myc; c-fos; p21; and cyclins A, B1, and D1
(41). In this study, we identified AREs in the 3’ UTR of AREG
mRNA as new targets of HuR. A prediction program for AREs
(46, 47) revealed that AREs are found in not only the 3’ UTR of
AREG mRNA but also those of other EGF family member
mRNAs, except for epigen. We actually examined the stabilities
of the mRNAs for EGF family ligands in keratinocytes after
UVB exposure and found that only AREG and HB-EGF mRNAs
were specifically stabilized in response to UV exposure (data
not shown). Because Hu protein family members do not stabi-
alyze all ARE-containing mRNAs in an indiscriminate manner
(43), HuR could interact with AREG mRNA in cooperation with
other coregulators depending on the cell type or physiological
state.

Several endogenous and external stimuli can alter HuR-me-
diated mRNA stability (40, 44, 45). As shown in Fig. 5, the sta-
bility of AREG mRNA was susceptible to inhibitors of EGFR
(AG1478 or an EGFR neutralizing antibody) and matrix metal-
loproteinases (KB-R7785). Given the fact that UV irradiation
promotes HuR activity by increasing the nuclear-to-cytoplas-
mic shuttling of HuR, downstream molecules of UV irradia-
tion-activated EGFR would act in a novel signaling pathway to
modulate HuR function (Fig. 7). It has been demonstrated that
UVB irradiation induces COX-2 expression by both transcrip-
tional and posttranscriptional regulation (46, 47). Moreover,
COX-2 is regulated by the ARE in the 3’ UTR of COX-2 mRNA
that controls both mRNA stability and protein translation (48),
resulting in the production of prostaglandins or thromboxanes
related to inflammation and carcinogenesis (34). Therefore,
one candidate regulator of HuR in the AREG mRNA stabiliza-
tion might be p38 MAPK because previous studies have implied
the critical participation of p38 MAPK in the regulation of
ARE-mediated stability of mRNAs such as COX-2 mRNA (34).
Besides the well known role of HuR in mRNA stabilization, it
can also enhance the translation of p53 and cytochrome c
mRNAs (49, 50). Further studies are needed to clarify the pre-
cise actions of HuR on AREG mRNA from the aspects of the
direct control of translation as well as mRNA stability.

In addition to keratinocytes, the presence of the autocrine
loop for EGF family ligand-EGFR signaling has been demon-
strated in a wide variety of cancers, including non-small cell
lung carcinoma, hepatocellular carcinoma, and breast cancer.
These findings suggest a correlation between autoinduction-
mediated autonomous cell growth and cancer phenotypes. The
expression of HuR is also correlated with a higher tumor grade
of breast, ovarian, and colorectal cancers (40). It was shown that
HuR protects cells against the death signaling induced by eto-
poside (VP-16), a widely used chemotherapeutic drug (51). Of
note, etoposide causes up-regulation of AREG mRNA and pro-
tein (52). In addition, although the involvement of HuR remains
elusive, etoposide increases the HB-EGF mRNA level through
its 3’ UTR (53). Because AREG and HB-EGF are known to pre-
vent cell death and might lower the efficacy of chemotherapy
(54), these observations suggest that HuR might enhance the
EGF family ligand-EGFR signaling and contribute to the gener-
ation of chemotherapy-resistant cells. Pharmacological strate-
gies aimed at specific inhibition of HuR could provide effective
anticancer therapies via the suppression of aberrantly expressed
AREG and HB-EGF.

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