Published in final edited form as:

Chem Biol Interact. 2016 June 25; 253: 38–47. doi:10.1016/j.cbi.2016.04.031.

Role of EGF Receptor Ligands in TCDD-induced EGFR Down-regulation and Cellular Proliferation

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Abstract

In cultures of normal human epidermal keratinocytes (NHEKs), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces the expression of the epidermal growth factor receptor ligands transforming growth factor- α (TGF- α) and epiregulin (EREG). TCDD also down-regulates EGF receptors (EGFR), suggesting that decreases in signaling contribute to the effects of TCDD. In this study, we treated post-confluent NHEKs with 10 nM TCDD and assessed its effects on EGFR binding, EGFR ligand secretion, basal ERK activity, and proliferation. TCDD caused time-dependent decreases in [¹²⁵I]-EGF binding to levels 78% of basal cell values at 72 h. Amphiregulin (AREG) levels increased with time in culture in basal and TCDD-treated cells, while TGF- α and epiregulin (EREG) secretion were stimulated by TCDD. Inhibiting EGFR ligand release with the metalloproteinase inhibitor batimastat prevented EGFR down-regulation and neutralizing antibodies for AREG and EREG relieved receptor down-regulation. In contrast, neutralizing TGF- α intensified EGFR down-regulation. Treating NHEKs with AREG or TGF- α caused rapid internalization of receptors with TGF- α promoting recycling within 90 min. EREG had limited effects on rapid internalization or recycling. TCDD treatment increased ERK activity, a response reduced by batimastat and the neutralization of all three ligands indicating that the EGFR and its ligands maintain ERK activity. All three EGFR ligands were required for the maintenance of total cell number in basal and TCDD-treated cultures. The EGFR inhibitor PD1530305 blocked basal and TCDD-induced increases in the number of cells labeled by 5-ethynyl-2'-deoxyuridine, identifying an EGFR-dependent pool of proliferating cells that is larger in TCDD-treated cultures. Overall, these data indicate that TCDD-induced EGFR down-regulation in NHEKs is caused by AREG, TGF- α , and EREG, while TGF- α enhances receptor recycling to maintain a pool of EGFR at the cell surface. These receptors are required for ERK activity, maintenance of total cell number, and stimulating the proliferation of a small subset cells.

Graphical abstract

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Conflict of interest

The authors declare that there are no conflicts of interest.

Keywords

2,3,7,8-tetrachlorodibenzo-*p*-dioxin; human keratinocytes; EGF receptors; extracellular signal-regulated kinase

1. Introduction

The epidermis is a stratified squamous epithelium that provides a barrier against the entrance of pathogens and foreign substances and protects the body from dehydration. Keratinocytes within the stratum basale are cuboidal, undifferentiated epithelial cells that divide and migrate superficially through the layers of the epidermis. Cells in the stratum basale maintain the proliferative capacity of the epidermis while the epidermal barrier is established as cells migrate outwardly and cornify [1]. As they progress upwards and differentiate, keratinocytes become more squamous-like [2,3], alter their membrane lipids, cross-link proteins and lose organelles such as mitochondria and nuclei [4–6]. The differentiation process is accompanied by decreases in epidermal growth factor receptor (EGFR) number at the cell surface (down-regulation), a reduction in EGFR signaling, and the attenuation of epidermal proliferative capacity [1,7–9], demonstrating the central role EGFRs play in the balance between proliferation in the basal layers and differentiation in the upper layers [10].

Keratinocytes express three members of the ErbB family of receptor tyrosine kinases, the EGFR (ErbB1), ErbB2, and ErbB3 (reviewed in [11]) with the EGFR appearing to be the most important member mediating ligand effects in these cells [12]. The fourth family member, ErbB4, is not expressed in skin [12]. Each ErbB receptor possesses differing ligand affinities, as well as overlapping, but distinct downstream signaling cascades and distribution within the epidermis [13]. EGFR activation leads to receptor dimerization and phosphorylation of tyrosine residues in the receptor's C terminus which provides docking sites for scaffolding and signaling molecules. All ErbB receptors can form homo- or heterodimers but EGFR homodimers engage cellular endocytic machinery leading to EGFR internalization and degradation, while ErbB heterodimers recycle to the cell membrane [14,15]. The EGFR is expressed in all epidermal layers [12], but its density is greatest in the stratum basale where it drives proliferation in cells that have lost contact with the matrix [16,17].

Cells expressing EGFRs typically produce EGFR ligands that maintain cells in the absence of exogenous growth factors [18–21]. Normal keratinocytes produce amphiregulin (AREG), epiregulin (EREG), transforming growth factor- α (TGF- α) and heparin binding EGF-like growth factor (HB-EGF) [11]. Amphiregulin is produced in the largest quantities, consistent with its ability to induce the strongest autocrine stimulation to cell growth [22]. In contrast, TGF- α , HB-EGF, and epiregulin are expressed at very low levels [23]. Keratinocyte production of TGF- α [24,25] and EREG [26] can also be increased by treating cells with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a response consistent with the ability of TCDD to stimulate proliferation. However, TCDD-mediated increases in TGF- α and EREG are also accompanied by accelerated differentiation [27–30] and down-regulation of keratinocyte EGFRs [30–32], and studies in our lab have shown that inhibiting EGFR activation promotes the differentiating effects of TCDD [28]. These data suggest that the loss of EGFR signaling mediates the accelerated differentiation of TCDD-treated keratinocytes.

Cellular responses to a ligand are determined by the number of receptors activated, and receptor down-regulation is a mechanism for modifying signaling dynamics to limit cellular responses [33,34]. The down-regulation of EGFRs observed as keratinocytes differentiate or in cells treated with TCDD may be an adaptive mechanism to maintain but redirect responsiveness in the face of continued agonist presence [35]. In mammary epithelial cells, Joslin *et al* [36] showed that local production of EGFR ligands reduced EGFR number, but elevated basal ERK activity and enhanced cell migration to levels greater than that produced by exogenous EGF. Thus, rather than preventing EGFR signaling, the local production of EGFR ligands by keratinocytes could fine tune EGFR signaling as receptor down-regulation is inversely proportional to receptor activity [37]. In this study, we used normal human epidermal keratinocytes (NHEKs) to study the mechanism involved in TCDD-induced EGFR down-regulation and the impact of down-regulation on EGFR signaling and cellular proliferation in post-confluent cultures. We show that TCDD increases the production of TGF- α and EREG, and that down-regulation of EGFRs is mediated by AREG, EREG and TGF- α . In addition, we show that TGF- α likely promotes receptor recycling to maintain ligand responsiveness. These changes in receptor availability are associated with ligand-dependent elevations in ERK activity as well as an increase in a small pool of proliferating cells.

2. Materials and methods

2.1. Cell Culture

In all experiments, fifth passage NHEKs from neonatal foreskins (Lonza, Mapleton, IL) were plated at 5,000 cells/cm² in Costar 24- or 96-well cell culture dishes (Corning, Corning, NY). Cells were maintained in keratinocyte serum-free medium (K-SFM; Gibco Invitrogen, Carlsbad, CA) containing 0.09 mM Ca, 5 ng/ml recombinant human EGF (EGF) and 50 μ g/ml bovine pituitary extract. The medium was changed every 48 h until confluence. At confluence, cells were changed to K-SFM without supplements (basal medium) for 48 h then transferred to basal medium containing TCDD (10 nM) as described in each figure legend. In some experiments, EGF (Bachem, Torrance, CA) was added to basal medium to

serve as a positive control for ligand-induced EGFR down-regulation. For immunofluorescence, NHEKs were plated in glass chamber slides (BD Biosciences, San Jose, CA) that had been coated with fetal bovine serum. The medium was changed every 72 h until confluence, at which time cells were treated for 72 hours as described above and then subjected to experimental protocols as described below.

2.2 EGFR ligand ELISAs

Culture medium was collected from basal and TCDD-treated cells 4–72 h after treatment and stored frozen in aliquots at -80°C until analyzed for AREG, HB-EGF, or TGF- α using ELISA kits from Abcam (Cambridge, MA) and EREG using a kit from Antibodies-Online Inc. (Atlanta, GA). Undiluted samples were added to the assays and ligand content was interpolated from standard curves. Data are reported in pg/ml and are the means $\pm$ SEM of three experiments assayed in duplicate.

2.3 Phosphoantibody Cell Based ELISA (PACE) and crystal violet staining

To test the effects of EGFR down-regulation on ERK activity, NHEKs grown in 96-well plates were treated for 72 h in the absence and presence of batimastat or EGFR ligand neutralizing antibodies as described below. At 72 h, ERK activity was measured by PACE assay as described by [38]. Briefly, treatments were terminated by rapid washing with ice-cold PBS and cells were fixed in 4% formaldehyde in PBS, blocked, and incubated overnight with an activation-specific rabbit monoclonal ERK antibody [phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204; 1:8000); Cell Signaling Technology, Danvers, MA]. The assays were developed by incubating with an HRP-conjugated goat anti-rabbit IgG (1:1000) and 1-Step Ultra TMB-ELISA substrate and read at 450 nm using a BioTek Synergy H1 microplate reader. To normalize to cell number, fixed cells were washed and stained with 0.04% crystal violet (Sigma Aldrich, St. Louis, MO) (w/v) in 4% ethanol following the protocol described by [39]. Cells were lysed overnight in 10% SDS and absorbance was measured at 595 nm. Data are expressed as a ratio of ERK (450 nm)/cell number (595 nm).

2.4. Interfering with ligand action

To investigate the role of each secreted ligand in EGFR down-regulation, ERK activation, and TCDD-induced proliferation, cells were grown in 96-well cell culture dishes in the presence or absence of 3 μM batimastat (Tocris Bioscience, Minneapolis, MN), a broad spectrum metalloproteinase (MMP) inhibitor [40], or neutralizing antibodies for TGF- α (5 $\mu\text{g}/\text{ml}$; R&D Systems, Minneapolis, MN), AREG (15 $\mu\text{g}/\text{ml}$; R&D Systems), or EREG (5 $\mu\text{g}/\text{ml}$; R&D Systems). The neutralizing capability of each antibody was verified by spiking basal medium with 0–30 ng/ml exogenous TGF- α , AREG, or EREG and preincubating with or without the appropriate amount of neutralizing antibody. Plates were then stopped after five minutes, and ERK activation was measured using a PACE assay. Anti-TGF- α , anti-AREG, and anti-EREG all effectively neutralized high doses of their respective ligands (Fig. 1S). Neutralizing antibodies were then added to binding assays, PACE assays and proliferation assays as described.

2.5 [¹²⁵I]-EGF and biotin-EGF Binding

Time-dependent loss of cell-surface EGFRs was determined in NHEKs grown to confluence in 24-well dishes (Corning, Corning, NY) and treated for 4–72 h in TCDD-containing medium. Following treatment, cells were washed three times with HEPES-buffered K-SFM. [¹²⁵I]-EGF/ml (specific activity 1128 Ci/mmol; Perkin Elmer, Waltham, MA) was diluted in HEPES-buffered K-SFM with 0.1 % bovine serum albumin (BSA) and binding reactions were initiated by adding 200 µL of [¹²⁵I]-EGF (250,000 cpm/ml) to each well in the presence or absence of excess (1 µg/ml) unlabeled EGF. Binding reactions were carried out for 60 min at 37°C and terminated by rapid washing with ice-cold phosphate-buffered saline (PBS). Monolayers were solubilized in 0.2N NaOH and aliquots were assayed for protein content using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA), and radioactivity by a Packard Cobra II Gamma counter (Perkin Elmer, San Jose, CA). Specific binding was determined by subtracting non-specific binding from total counts bound and normalized to protein content. Data are reported as the percentage of binding observed in time-matched controls and are means ± SEM of three separate binding experiments assayed in triplicate.

In order to conserve antibody and increase assay throughput while assessing the role of EGFR ligands in receptor down-regulation, cells were grown in 96-well dishes treated with EGF or TCDD for 72 h in the presence of batimastat or neutralizing antibodies as described above. Medium was spiked with batimastat or neutralizing antibody after 36 h of treatment. The effect of interfering with EGFR ligand availability on cell-surface EGFRs was determined using biotin-EGF (Invitrogen, Carlsbad CA) in a modified protocol from de Wit *et al* [41]. Plates were washed in ice-cold PBS and incubated for 1 h on ice with 10 ng/ml biotin-EGF in the presence or absence of 1 µg/ml unlabeled EGF. Cells were fixed for 30 min at 37°C in 4% formaldehyde, washed once with PBS, followed by two five-min washes in PBS containing 50 mM glycine. Cells were blocked in 2% gelatin in PBS with 0.1% Triton X-100 (PBS-T) for 1 h at 37°C, then incubated with streptavidin-HRP at a 1:500 dilution (Cell Signaling Technology, Danvers, MA) in 0.2% gelatin in PBS-T for 1 h at 37°C. Plates were subjected to three five-min washes with PBS-T and two five-min washes with PBS before developing with 1-Step Ultra TMB-ELISA Substrate (Thermo Fisher, Waltham, MA). Absorbance at 450 nm was read on a BioTek Synergy H1 microplate reader and normalized to crystal violet absorbance at 595 nm. Data are reported as the percentage of binding in basal cells. To assess the ability of each ligand to down-regulate EGFRs, post-confluent NHEKs were exposed to EGF, TGF-α, amphiregulin, or epiregulin for 5–120 min using concentrations equal to the maximum amount of that ligand in the cultures at 3 days. Cells were then placed on ice and cell surface receptors were analyzed by measuring biotin-EGF binding as described above.

2.6 Double-stranded DNA analysis

As our previous study shows that TCDD treatment compromises mitochondrial function [42], analysis of changes in cell number was measured by double stranded DNA labeling using the FluoReporter blue fluorometric dsDNA Quantitation kit (Molecular Probes, Inc, Eugene, OR), following the manufacturer's instructions. Briefly, cells were grown to confluence in clear bottom black well plates (Corning Costar, Corning NY) and treated with

10 nM TCDD in the absence or presence of 3 μ M batimastat or neutralizing antibodies. At 72 hours, medium was removed from the cells, and the plates were frozen at -80°C to lyse cells and release DNA. Plates were then thawed and rehydrated with Hoechst 33258 in Tris-NaCl-EDTA buffer and fluorescence was measured on a BioTek Synergy H1 microplate reader with excitation and emissions at 360 and 460 nm, respectively. Cell number in each treatment group was determined from a standard curve plated at the start of the experiment.

2.7 EdU Labeling

At confluence, cells grown in glass chamber slides were treated for 72 h with 10 nM TCDD in the presence or absence 300 nM PD153035 (EMD Millipore, Billerica, MA), a highly selective EGFR inhibitor. Sixteen hours before the end of the experiment, cells were incubated with 10 μ M 5-ethynyl-2'-deoxyuridine (EdU), fixed with 4% formaldehyde in PBS then permeabilized with 0.5% Triton-X 100 in PBS. Slides were incubated with the Click-It reaction cocktail containing copper sulfate and an AlexaFluor 647-conjugated azide that was crosslinked to cellular EdU according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Slides were coverslipped using DAPI-containing mounting medium to stain nuclei. Cells were imaged at 20 $\times$ magnification on a Nikon A1 confocal microscope (Nikon, Inc, Melville, NY) at 647 nm for EdU-positive nuclei and 405 nm for total nuclei. Identical capture parameters were used for all images. EdU positive nuclei as well as total (DAPI-labeled) nuclei were counted using CellProfiler version 2.1.0 (Broad Institute, Cambridge, MA). Images were converted to gray scale then counted via "count objects" selecting for circularity and filtering using a Mixture of Gaussian (MoG) Global correction with standard threshold correction factors, and upper and lower bounds (1.0, 0.0, 1.0, respectively) [43]. The experiment was performed three times, with three slides per experiment. Five random fields on each of 3 slides were counted for all treatments and conditions.

2.8. Data analyses

EGFR binding data and PACE assays were performed in three experiments utilizing triplicate wells. Data were analyzed via two-way ANOVA and significance ($p < 0.05$) was determined using a Bonferroni post-hoc test as performed in Prism GraphPad software version 5.0 (La Jolla, CA). TGF- α , HB-EGF, AREG and EREG ELISAs were performed with culture medium collected from three experiments and assayed in duplicate. EGFR ligand concentrations were determined from standard curves and analyzed via one- or two-way ANOVA with a Bonferroni post-hoc test, as appropriate.

3. Results

3.1. EGFR down-regulation in TCDD-treated cells

Previous studies have shown that treating NHEKs for 1–4 days with TCDD causes the loss of cell-surface EGFRs [30–32]. To determine how rapidly this response occurs and to compare it to ligand-induced down-regulation by EGF, we performed radioligand binding assays in NHEKs. EGF (100 ng/ml) rapidly reduced [^{125}I]-EGF binding to roughly 10% of control values by 4 h, maintaining this significant ($p < 0.05$) level of inhibition for the duration of the experiment (Fig. 1A). In contrast, TCDD treatment had a limited effect on

[¹²⁵I]-EGF binding during the first 24 h but by 48 h, binding had decreased to 64% of control. At 72 h, binding rebounded to 78% of control values, but was still significantly lower than that in time-matched cells. In no case was the reduction in binding in TCDD-treated cells as rapid or as complete as that observed with EGF treatment. The decrease in [¹²⁵I]-EGF binding observed in EGF- or TCDD-treated cells was not the result of time-dependent decreases in basal cell receptor number, as binding did not change significantly with time in culture (Fig. 2S).

To determine if lower doses of EGF could mimic the levels of down-regulation observed in TCDD-treated cells, NHEKs were treated with EGF (0.1–100 ng/ml) followed by the measurement of [¹²⁵I]-EGF binding. In cells treated with 0.1 ng/ml EGF, [¹²⁵I]-EGF binding was reduced to 90% of control within 24 h while 1 ng/ml EGF reduced binding to 60% of control within 12 h. By 72 h, [¹²⁵I]-EGF binding had returned to control levels in cells treated with either EGF concentration. Increasing the EGF dose to 10 or 100 ng/ml produced a more rapid, extensive, and persistent reduction in [¹²⁵I]-EGF binding, reaching ~40% of control levels by 72 h for 10 ng/ml and 12% of control values for 100 ng/ml (Fig. 1B). These data show that the degree of receptor loss is dose-dependent and low concentrations of EGF remove fewer receptors from the cell surface.

3.2. Effect of TCDD on EGFR ligand secretion

Keratinocytes secrete several EGFR ligands (see Pastore et al [¹¹]) with TCDD reported to increase the expression and production of TGF- α [^{24,25}], and AREG [⁴⁴]. Our observation that TCDD treatment decreases [¹²⁵I]-EGF binding (Fig. 1) suggested that the local production of an EGFR ligand(s) may drive receptor down-regulation. To assess the impact of TCDD on EGFR ligand secretion, we measured AREG, EREG, TGF- α , and HB-EGF in culture medium over the course of 72 hours. These ligands were chosen based upon the literature, their ties to epidermal disease and homeostasis, and evidence of TCDD-induced changes in mRNA expression by RT-PCR or microarray [⁴²]. AREG secretion increased with time in culture, was comparable in both basal and TCDD-treated cells, and was inhibited by the broad-spectrum MMP inhibitor batimastat (Fig. 2A,D). EREG secretion was minimal (26 pg/ml) in basal cells, but at 72 h was present at a concentration of 718 pg/ml in culture medium from TCDD-treated cells (Fig. 2B). Like AREG, EREG production was inhibited by batimastat (Fig. 2E). Batimastat-sensitive TGF- α secretion by basal cells increased by 65 % at 72 hours and TCDD significantly enhanced TGF- α production over basal cells at 72 hours treatment (Fig. 2C, F). At 72 h, the concentration of TGF- α in the medium from basal cells was 119 pg/ml, versus 428 pg/ml in TCDD-treated cells. This value is comparable on a molar level to ~ 0.5 ng/ml EGF, a value between the low doses of EGF (0.1 and 1 ng/ml) that produce modest decreases in [¹²⁵I]-EGF binding in Fig. 1B. HB-EGF concentrations were below the detection limits of our ELISA (data not shown), consistent with the low levels of HB-EGF produced by NHEKs in culture [⁴⁵] and its primary role in wound healing and keratinocyte migration [⁴⁶]. Together, the data in Fig. 2 indicate TCDD modifies both the ligands produced and the concentrations available to the cells.

3.3 Role of EGFR ligands in EGFR down-regulation

The data presented in Fig. 2 show that NHEKs secrete AREG, EREG, and TGF- α , with TCDD enhancing the production of EREG and TGF- α . These data suggest that an EGFR ligand(s) causes the loss of [125 I]-EGF binding observed in TCDD-treated cells. To assess the role of these ligands in mediating TCDD-dependent EGFR down-regulation, NHEKs were treated with TCDD or EGF in the presence or absence of batimastat (3 μ M) or neutralizing antibodies for AREG, EREG, or TGF- α , alone or in combination. Consistent with [125 I]-EGF binding (Fig. 1A), 10 nM TCDD and 10 ng/ml EGF significantly reduced biotin-EGF binding at 72 h with TCDD decreasing binding to $44 \pm 11\%$ and EGF to $38 \pm 19\%$ of basal cells (Fig. 3A). Batimastat relieved receptor down-regulation in TCDD-treated cells, indicating that the release of soluble EGFR ligands was driving receptor loss from the cell surface. As expected, batimastat had no effect on receptor down-regulation produced by the addition of exogenous EGF.

To examine the individual roles of the EGFR ligands in receptor down-regulation, NHEKs were treated with neutralizing antibodies for AREG, EREG, or TGF- α . TCDD treatment reduced biotin-EGF binding to $43 \pm 7\%$ of basal control values (Fig. 3B), and neutralizing AREG or EREG returned binding to levels that were not significantly different from basal control cells ($67 \pm 13\%$ and $61 \pm 10\%$ of basal control levels, respectively). These data indicate that both AREG and EREG contribute to EGFR down-regulation in TCDD-treated cells. In contrast, neutralizing TGF- α in TCDD-treated cells significantly enhanced EGFR down-regulation ($11 \pm 1\%$ of basal control levels), suggesting that TGF- α promotes receptor recycling rather than down-regulation. When TCDD-treated cells were incubated with all three neutralizing antibodies, biotin-EGF binding remained at levels about 30 % of basal, indicating that the effect of neutralizing TGF- α predominates at 72 hours post-confluence.

The enhanced down-regulation of EGFRs when TGF- α was neutralized suggested that TGF- α may alter EGFR trafficking in TCDD-treated cells. Since the fate of an internalized receptor has a substantial impact on the duration of a receptor signal [36,47], we examined the effect of pretreating NHEKs with EGF, AREG, EREG or TGF- α on biotin-EGF binding. Although exogenous delivery of ligands does not precisely reflect the impacts of autocrine delivery of EGFR ligands [36], we assessed the ability of AREG, EREG, and TGF- α to down-regulate and recycle EGFRs. NHEKs were treated for 5–120 min with concentrations of exogenous EGFR ligands comparable to the levels found in conditioned medium at 72 h, then the amount of biotin-EGF binding was assessed. As observed in Fig. 1, treatment with EGF caused a rapid reduction in biotin-EGF binding to $11 \pm 4\%$ of T_0 values, a response that was sustained throughout the experiment (Fig. 4). TGF- α reduced binding to 87% of T_0 values within 10 min, while AREG reduced binding to 63% of T_0 values by 15 min. EREG did not affect biotin-EGF binding at any time point. These data are consistent with all three ligands producing effects on EGFR trafficking distinct from those of EGF, and suggest that one or all maintain a population of EGFRs at the cell surface. The effects of TGF- α in particular suggest that this ligand promotes recycling and EGFR synthesis.

3.4. Effect of EGFR ligands on ERK activity

Human keratinocytes maintain high steady-state levels of ERK activity due to the autocrine production of EGFR ligands [48] and the continued presence of EGFRs (Fig 3B) could be important in maintaining ERK activity. In fact, ERK activity was elevated in TCDD-treated cells as early as 24 h after the start of the treatment even in the face of a reduction in the number of EGFRs, and was significantly greater than time-matched controls at 72 h (Fig. 5A). To determine which EGFR ligand(s) enhanced ERK activity, we treated cells for 72 h with TCDD in the absence or presence of batimastat (3 μ M) or neutralizing antibodies for AREG, EREG, or TGF- α alone, or in combination. Batimastat reduced ERK activity to 52% and 55% of control levels in both basal and TCDD-treated cells, respectively (Fig. 5B). These data indicate that the residual EGFRs present on the cell surface are sufficient to maintain EGFR-dependent ERK activity in response to locally secreted EGFR ligands in both basal and TCDD-treated cells. When we examined the roles of each EGFR ligand in maintaining ERK activity, neutralizing an individual ligand had no impact on the elevated ERK activity observed in TCDD-treated cells. However, when NHEKs were incubated with all three neutralizing antibodies, ERK activity was reduced to levels that were not significantly different from untreated basal cells (Fig. 5C). These data indicate that all three ligands are required to produce the increase in ERK activity observed in TCDD-treated cells.

3.5. Role of EGFR ligands in regulating cell number

The presence of AREG, EREG, and TGF- α in NHEK culture medium and the observation that all three ligands have an effect on EGFR down-regulation as well as maintenance of ERK activity led us to examine the role of each ligand plays in the proliferative response reported in TCDD-treated cells [25,26,49]. Cell numbers at 72 hours were not different in basal and TCDD-treated cells (95713 ± 2251 vs $94,360 \pm 3175$, respectively; Fig. 6A). These results are not surprising as these are post-confluent cultures. Neutralizing each individual ligand produced modest reductions in cell number, with the greatest effect occurring in basal and TCDD-treated cells treated with the AREG antibody (a 6 % drop from 95714 ± 2251 to 90314 ± 650 cells in basal cultures and a 7 % drop from 94380 ± 3175 to 88027 ± 1412 cells in TCDD-treated cultures). While the loss of individual ligands was not statistically significant, each produced modest reductions in cell number and neutralizing all three ligands reduced cell number significantly in both basal (10.3%) and TCDD-treated (11.3%) cells. These data indicate that all three EGFR ligands play a role in maintaining cell number in post-confluent monolayers of basal and TCDD-treated cells.

Since TCDD produced no significant increase in cell number compared to basal cells, we wondered whether there might be a small population of cells proliferating in response to TCDD. To address this question, we measured DNA synthesis using EdU labeling in cells treated for 72 h with TCDD in the presence or absence of the EGFR inhibitor PD153035 (300 nM). Compared to basal cells, TCDD treatment significantly increased the percentage of the cell population that was proliferating (Fig. 6B,C). Inhibiting EGFR activity with PD153035 significantly reduced this percentage in both basal and TCDD-treated cells. The increased percentage of EdU positive nuclei in TCDD-treated cells in the face of a total cell population comparable to that of basal cultures, suggests that TCDD-treated cultures have a larger proliferative capacity than basal cells even in these post-confluent cultures.

4. Discussion

The epidermis regenerates constantly, requiring the concurrent expression of proliferative programming in the basal layer and differentiation programming in the suprabasal layers [16,17]. The EGFR and its ligands play critical roles in regulating the balance between proliferation and differentiation. EGFR number is greatest in the basal layer of the epidermis where its activation promotes proliferation and declines as keratinocytes migrate suprabasally and undergo terminal differentiation [16,17]. The EGFR and its ligands establish an autocrine loop in the skin with receptor activation stimulating cell proliferation in the basal layer, and with decreased receptor number and activity leading to differentiation as the cells migrate suprabasally [11,16,17]. The environmental toxin TCDD alters this autocrine loop by promoting EGFR down-regulation [30–32] and enhancing the production of receptor ligands [24–26]. These apparently opposing responses have been used to explain the proliferative effects of TCDD (increased EGFR ligand production) and its ability to accelerate keratinocyte differentiation (EGFR down-regulation and loss of signaling). However, *in vitro* studies have shown that the increase in ligand production occurs in the face of reduced receptor number, an effect that should alter the cells' ability to respond to the ligands. Since cellular responses to an agonist reflect the number of receptors activated [37], we were interested in understanding how a decrease in receptor number accompanied by increased ligand production might mediate the effects of TCDD in NHEKs. Using post-confluent NHEKs, we show that TCDD increases the production of TGF- α and EREG, and that down-regulation of EGFRs is mediated by AREG, EREG and TGF- α . In addition, we show that TGF- α promotes receptor recycling. These changes in receptor availability are associated with an increase in steady-state ERK activity that is ligand-dependent, as well as an increase in a small pool of EGFR-dependent proliferating cells which maintains total cell number.

Basal NHEKs increase their AREG production while producing only small amounts TGF- α and virtually no EREG. Since the medium of basal cells are not supplemented with growth factors, these data indicate that autocrine production AREG is maintaining these cells [18–21]. These data are consistent with the observations of Piepkorn *et al* [22] who showed that AREG is the most abundant and efficacious regulator of keratinocyte growth. TCDD-treated keratinocytes produced comparable amounts of AREG but had elevated production of both TGF- α and EREG, responses also observed by Choi *et al*. [24] and Patel *et al*. [26]. These changes in the production all three ligands occur at the same time that receptor numbers are declining, arguing that one or more of these ligands mediates EGFR down-regulation in TCDD-treated cells. When we inhibited EGFR ligand release with the MMP inhibitor batimastat, receptor binding increased not only in TCDD-treated cells but also in basal cells, arguing that a ligand(s) is modulating the number of cell surface receptors in both cell populations. Subsequent experiments with ligand-neutralizing antibodies showed that the elimination of all three ligands reduced receptor number in both basal and TCDD-treated cells. This was somewhat surprising as we expected the removal of all the ligands to relieve receptor downregulation. In fact, neutralizing either AREG or EREG in TCDD-treated cells partially restored biotin-EGF binding; however eliminating TGF- α intensified receptor loss, a response also observed in basal cells. These data suggest that TGF- α is required to

maintain a pool of receptors at the cell surface, a response consistent with the ability of TGF- α to bias EGFRs to the cell membrane [47,50]. In fact, our observation that exogenous TGF- α increases EGFR binding at the cell surface supports this role of TGF- α , although these data must be interpreted cautiously as exogenous addition of ligands does not precisely reflect the impacts of autocrine of EGFR ligands [36]. TGF- α -induced increases in cell membrane-associated EGFRs have also been observed in prostate cancer cells where the ligand stabilizes EGFR mRNA and enhances the *de novo* synthesis of the receptor [51]. Vassar and Fuchs [52] showed that there were no major differences in EGFR levels in TGF- α -overexpressing mice compared to normal, demonstrating that TGF- α does not promote the loss of EGFRs. Together these results demonstrate that the combined effects of the three ligands is to maintain a reduced number of receptors on the cell surface by causing down-regulation (AREG, EREG, and TGF- α) and promoting recycling (TGF- α).

Receptor down-regulation is typically thought of as a way for cells to modify their responses to continued agonist presence. Differences in signaling dynamics (signal intensity, frequency, and duration) can encode specific cellular responses (34), as exemplified by studies from Joslin *et al.*, [36] who showed that the autocrine production of EGFR ligands leads to a persistent reduction in EGFR number but a sustained elevation in ERK activity that mediated an enhanced migratory response compared to that produced by exogenous EGF. Likewise, we show that the TCDD-induced down-regulation of EGFRs in NHEKs was accompanied by an elevation in ERK activity that was significantly greater than that in basal cells and maintained by the autocrine production of AREG, EREG, and TGF- α . The production of EGFR ligands also maintained ERK activity in basal cells, consistent with the findings of Iordanov *et al.* [48]. In addition, these data indicate that TCDD alters an autocrine loop already present in keratinocytes by reducing receptor number with AREG, EREG, and TGF- α and maintaining cellular responsiveness to secreted ligands by TGF- α -induced receptor recycling.

Increases in ERK activity mediate a host of cellular responses including proliferation [53]. Both basal and TCDD-treated cells grow in the absence of exogenous growth factors, reflecting the ability of locally produced EGFR ligands to activate ERK and maintain total cell number. However, the total number of cells in basal and TCDD-treated cultures were not significantly different. This result was not surprising as our monolayer cultures are post-confluent and more reflective of intact skin. Neutralizing individual ligands had modest impacts on cell number in basal or TCDD-treated cells, but in both basal and TCDD-treated cells the response was greatest in cells treated with AREG antibodies, consistent with AREG being the most abundant and efficacious regulator of keratinocyte growth [22]. Cell number was significantly reduced in basal and TCDD-treated cells when all three ligands were neutralized. Since NHEKs stratify in this culture system, our data suggest that the EGFR ligands are required for the homeostatic replacement of cells lost to sloughing during the culture period in both basal and TCDD-treated cells. However, the complement and concentrations of EGFR ligands produced by basal and TCDD-treated cells was different; AREG and modest amounts of TGF- α are produced in basal cells while TCDD-treated cells produced AREG, greater amounts of TGF- α , and EREG. Since hyperproliferation is a well-described response to TCDD *in vitro* [25,26,49] and *in vivo* [44], we asked whether or not the TCDD could enhance the number of proliferating cells in the culture. EdU labeling of

basal and TCDD-treated cells indicated that both populations required a functional EGFR to maintain a small pool of proliferating cells. In addition, TCDD-treated cells, which have a higher concentration of secreted TGF- α compared to control cells and produce EREG, show a greater percentage of EdU positive cells, indicating that these cultures have a greater proliferative capacity. These data support the idea that the hyperproliferative effect of TCDD in NHEKs is the result of producing a larger population of proliferative cells within a differentiating epidermis. Maintenance of this proliferative pool in basal cells reflects the ability of AREG to promote proliferation while the small amounts of TGF- α produce by these cells maintains receptor number and ligand responsiveness. AREG and TGF- α would serve the same purpose in TCDD-treated cells, but the enhanced production of TGF- α and EREG is responsible for the increased size of the proliferative pool of cells in these cultures.

In summary, our data indicate that TCDD alters an autocrine loop present in keratinocytes by enhancing the production of EGFR ligands and modifying EGFR trafficking to maintain the cells' responsiveness to locally produced growth factors. The alteration in EGFR number in TCDD-treated cells required AREG, EREG, and TGF- α and is accompanied by a TGF- α -mediated recycling of receptors that maintains a pool of receptors at the cell surface. This receptor pool preserves the cell's ability to respond to the local effects of AREG, EREG, and TGF- α , leading to a sustained elevation in basal ERK activity and the maintenance of a pool of proliferating cells that can quickly respond to disruption of the culture and replace cells lost to sloughing of the stratifying culture (Fig. 7).

5. Conclusion

TCDD treatment of NHEKs produces a ligand-dependent decrease in EGFR number that is limited by the ability of TGF- α to promote receptor recycling. These ligands and receptor changes enhance the activity of the autocrine loop that sustains the monolayer with TGF- α promoting EGFR recycling and upregulation as well as sustained levels of ERK activity that promote homeostatic replacement of cells during the culture period.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported in part by NIH grant R01 ES017014. The funding agency had no role in the design or interpretation of the data in this study.

Abbreviations

AREG	Amphiregulin
BTC	Betacellulin
Ca	Calcium
EdU	5-Ethynyl-2'-deoxyuridine

EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay
EREG	Epiregulin
ERK	Extracellular Signal-Related Kinase
HB-EGF	Heparin Binding EGF-like Growth Factor
K-SFM	Keratinocyte Serum Free Medium
NHEK	Normal Human Epidermal Keratinocyte
MMP	metalloproteinase
PACE	Phosphoantibody Cell Based ELISA
TCDD	2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin
TGF-α	Transforming Growth Factor-alpha

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Highlights

- TCDD alters an autocrine loop present in keratinocytes by enhancing the production of EGFR ligands and modifying EGFR trafficking
- TCDD-induced EGFR down-regulation in NHEKs reflects the combined effects of AREG, EREG, and TGF- α
- TGF- α maintains a pool of EGFR at the cell surface
- AREG, EREG, and TGF- α produce a sustained elevation in ERK activity
- AREG, EREG, and TGF- α maintain of a pool of proliferating cells

Fig. 1. TCDD decreases [¹²⁵I]-EGF binding. NHEKs were grown as described in the Material and methods section and (A) treated for the times indicated with EGF (100 ng/ml) or TCDD (10 nM), then [¹²⁵I]-EGF binding was determined. Data are reported as the means ± SEM of three experiments assayed in triplicate. * indicates p 0.05 for treatment effect compared to time-matched control (B) NHEKs were treated for the times indicated with increasing concentrations of EGF (0.1–100 ng/ml), then [¹²⁵I]-EGF binding was determined. Data means ± SD of one experiment assayed in triplicate. The dashed line in (A) and (B) indicates 100%.

Fig. 2. Effect of TCDD on MMP-dependent EGFR ligand secretion. NHEKs were treated for 2–72 h with TCDD (10 nM) and the culture medium was collected for the measurement of EGFR ligands. (A) AREG, (B) EREG or (C) TGF-α content were determined by ELISA as described in Materials and methods section. Data are means ± SEM of three experiments assayed in duplicate. * indicates significantly different from time-matched control (TMC), $p < 0.05$. In D–F, cells were treated for 72 h with vehicle or TCDD ± 3 μM batimastat (bat).

The culture medium was collected at 72 h for the measurement of **(D)** AREG, **(E)** EREG, or **(F)** TGF- α . ND indicates not detectable.

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Fig. 3.

Reducing EGFR ligand availability modifies EGFR down-regulation in response to TCDD. (A) NHEKs were treated with or without TCDD (10 nM) or EGF (10 ng/ml) for 72 h in the absence or presence of batimastat (bat; 3 μ M) or (B) neutralizing antibodies for AREG (15 μ g/ml), EREG (5 μ g/ml), TGF- α (5 μ g/ml), or all three growth factors (anti-all-GF) for 72 h. The measurement of biotin-EGF binding was then determined as described in the Material and methods section. Basal refers to medium (K-SFM) without TCDD; control refers to treatments in the absence of batimastat or neutralizing antibodies. Anti-all-GF refers to cells in the presence of neutralizing antibodies for AREG, EREG, and TGF- α . Data are means $\pm$ SEM of six experiments assayed in triplicate, normalized to crystal violet staining, and reported as the % biotin-EGF bound in basal control cells. (a) indicates significantly

different from the basal control at $p = 0.05$. * indicates significantly different from the within treatment control ($p = 0.05$).

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Fig. 4.

EGFR ligands differentially alter EGFR recycling. NHEKs were treated for 0–120 min with 60 ng/ml EGF and concentrations of ligands present in culture medium at 72 h (3000 ng/ml AREG, 412 ng/ml TGF- α , or 627 ng/ml EREG) then biotin-EGF binding was measured on ice to prevent internalization. The amount of biotin-EGF bound was then determined as described in the Materials and methods section. Data are means $\pm$ SEM of triplicate experiments. (a) indicates significantly different from T₀ ($p < 0.05$). The dashed line represents biotin-EGF binding at T₀.

Fig. 5.

TCDD-mediated increases in ERK activity are EGFR ligand-dependent. To assess the effect of TCDD on basal ERK activity over 72 h, NHEKs were grown for 0–72 h with or without 10 nM TCDD and (A) basal ERK activity was measured at the indicated times as described in the Materials and methods section. Data are reported as % time matched control and are means $\pm$ SEM of three experiments assayed in triplicate. * indicates significant difference from the time-matched control ($p < 0.05$) (B) To assess the role of secreted ligands on ERK activity, NHEKs were treated for 72 h with TCDD in the presence or absence of batimastat (bat; 3 μ M) or (C) neutralizing antibodies for AREG (15 μ g/ml), EREG (5 μ g/ml), TGF- α (5 μ g/ml), or all three growth factors (anti-all-GF). Basal refers to medium (K-SFM) without TCDD; control refers to treatments in the absence of inhibitors. Data are means $\pm$ SEM of three experiments assayed in triplicate. In B, C, * indicates significantly different from TCDD alone ($p < 0.05$) and (a) indicates significantly different from basal control ($p < 0.05$). The dashed line indicates the basal control normalized to 100%.

Fig. 6. Reducing ligand availability affects cell number and proliferation. **(A)** NHEKs were grown in the presence or absence of neutralizing antibodies for AREG (15 µg/ml), EREG (5 µg/ml), TGF-α (5 µg/ml) or all three growth factors (anti-all-GF) antibodies. After treatment cell number was measured as described in the Materials and methods section. Basal refers to medium (K-SFM) without TCDD; control refers to treatments in the absence of neutralizing antibodies. Data are means ± SEM of three separate experiments assayed in triplicate. * indicates significantly different from within treatment control. **(B)** and **(C)**, NHEKs were grown to confluence in glass chamber slides and then treated for 72 h with TCDD (10 nM) in the presence or absence of PD153035 (300 nM), an EGFR inhibitor. During the last 16 h cells were incubated with 10 µM EdU to label proliferating cells. **(B)** Representative confocal images of total (DAPI; blue) and EdU-staining nuclei (pink) at 20× magnification. Scale bar indicates 20 µm Basal refers to medium (K-SFM) without TCDD; control refers to treatments in the absence of PD153035. **(C)** Proliferative index is determined as the percentage of the total cell population labeled by EdU [(EdU labeled/DAPI labeled) × 100]. Data are means ± SEM from counting five fields from three separate experiments. * indicates significantly different from the within treatment control (p < 0.05). (a) indicates significantly different from basal control (p < 0.05).

Fig. 7.

TCDD alters the EGFR/ligand autocrine loop present in keratinocytes. **(A)**, TCDD-induced EGFR internalization in NHEKs reflects the combined effects of AREG, EREG and TGF- α , while TGF- α promotes EGFR recycling. This receptor pool preserves the cell's ability to respond to the local production of AREG, EREG, and TGF- α leading to a sustained elevation in ERK activity compared to basal cells. **(B)** The enhanced production of TGF- α and EREG enhances the proliferation of a small subset cells in the basal or suprabasal layers of the skin that serve to maintain total cell number.