Local cortisol activation is involved in EGF-induced immunosuppression

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ABSTRACT

The major effects of the epidermal growth factor receptor (EGFR) signalling pathway on keratinocytes are cell proliferation, cell differentiation, and wound healing. In addition to these effects, an immunosuppressive effect of EGFR signalling has been reported. However, the precise mechanism of immunosuppression by EGFR signalling is not well understood.

In this study, we clarified the involvement of increased local cortisol activation in EGFR signalling-induced immunosuppression in keratinocytes. EGF treatment up-regulated the expression of 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) and supernatant cortisol levels in a dose-dependent manner in keratinocytes. 11β-HSD1 is an enzyme that catalyses the conversion of cellular hormonally inactive cortisone into active cortisol. qRT-PCR and ELISA assays indicated that EGF significantly decreased tumour necrosis factor α (TNF-α) induced interleukin-6 (IL-6) expression in keratinocytes. Similarly, 11β-HSD1 overexpression significantly decreased TNF-α induced IL-6 expression. We evaluated the role of 11β-HSD1 in immunosuppression through EGFR signalling. Blockade of 11β-HSD1 via 11β-HSD1 inhibitor reversed both the expression and production of TNF-α induced IL-6, which was decreased by EGF in keratinocytes. Therefore, increased local cortisol activation by 11β-HSD1 is involved in EGFR signalling-induced immunosuppression in keratinocytes.

Finally, we evaluated whether EGFR inhibition by cetuximab affects the expression of 11β-HSD1. We found that 0.1 μg cetuximab decreased 11β-HSD1 transcript levels in keratinocytes. The changes in 11β-HSD1 were more apparent in TNF-α treated cells.

As 11β-HSD1 expression in keratinocytes is associated with inflammation and cell proliferation, this mechanism may be associated with adverse skin reactions observed in patients treated with EGFR inhibitors.

Introduction

The epidermal growth factor receptor (EGFR) signalling pathway is essential for skin development and homeostasis. A well-characterized biological effect of the EGFR signalling pathway in the skin is to regulate keratinocyte proliferation. EGFR signalling also induces epithelial mesenchymal transition in keratinocytes. Furthermore, an immunosuppressive effect of the EGFR signalling pathway has been reported. Uregulation of EGFR and its ligands were found in atopic dermatitis, and EGFR signalling attenuated development of atopic dermatitis in a murine model. A homozygous, loss-of-function, missense mutation in EGFR was reported in a patient with lifelong inflammation affecting the skin, bowel, and lung.

Cortisol-activating enzyme 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) catalyses the conversion between hormonally active cortisol/corticosterone and inactive cortisone/11-dehydrocorticosterone (11-DHC) in cells. The 11β-HSD1 isoform is predominantly a reductase that catalyses conversion of cortisone/11-DHC to cortisol/corticosterone. The 11β-HSD2 isoform catalyses inactivation of cortisol/corticosterone to cortisone/11-DHC. Circulating cortisol is converted to active cortisol in tissue through 11β-HSD1 in cells.

Glucocorticoid inhibits wound healing by inhibiting keratinocyte migration. We have been investigating the function of 11β-HSD in skin and found that it regulates cell proliferation and cutaneous wound...
healing.\textsuperscript{9} 11\textbeta-HSD is also associated with skin diseases such as skin cancer and psoriasis.\textsuperscript{10,11} In skin inflammation, we found that various stimuli, such as UVB irradiation and hapten application, increase the level of 11\textbeta-HSD1 in skin and in keratinocytes.\textsuperscript{11-14} In addition, low-dose hapten-induced irritant dermatitis and UVB-induced dermatitis were augmented in keratinocyte-specific \textit{Hsd11b1} knockout mice.\textsuperscript{11} Thus, local cortisol activation through 11\textbeta-HSD1 is considered to have a local immunosuppressive effect.

In this study, we investigated the mechanism of EGFR signalling-induced immunosuppression. We found that activation of local cortisol through 11\textbeta-HSD1 is important in EGFR signalling-induced immunosuppression in keratinocytes.

**Materials and methods**

**Materials**

Cortisone (catalog no. C2755) and cortisol (catalog no. H0888) were purchased from Sigma-Aldrich. EGF (catalog no. 236-EG) and tumour necrosis factor \textalpha (TNF-\textalpha) (catalog no. 210-TA) were purchased from R&D Systems. Cetuximab was purchased from Merck.

**Cell culture**

Normal epidermal human keratinocytes (NHEK) from a single donor were purchased from DS Pharma Biomedical. NHEKs were cultured on type-1 collagen plates (IWAKI, catalog no. 4815-010) in Epilife medium (Invitrogen, catalog no. MEPI500CA) until 70–90% confluent. Passage 3 or passage 4 cells were used for experiments.

**HSD11\textbeta1 expression vector construction**

A mammalian expression vector encoding HSD11\textbeta1 (HSD11\textbeta1/pBApo-CMV Neo DNA) was constructed by inserting human HSD11\textbeta1 cDNA into pBApo-CMV Neo DNA (Takara Bio Inc.). NHEKs (100,000 cells/ml) were seeded on type-1 collagen-coated plates 1 d prior to transfection. Cells were transfected with HSD11\textbeta1/pBApo-CMV Neo DNA or control vector at 500–1000 ng/ml using Lipofectamine LTX (Invitrogen, catalog no. 94756) and PLUS Reagents (Invitrogen, catalog no.10964-021) according to the manufacturer’s instructions. The culture medium was replaced after 6 h.

**RNA isolation and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)**

Total RNA was isolated from cells using a Maxwell\textsuperscript{®} 16 LEV Simply RNA Tissue kit (Promega, catalog no. AS1280). The product was reverse-transcribed into first-strand cDNA. HSD11\textbeta1 and IL-6 expression was measured using THUNDERBIRD SYBR qPCR Mix (TOYOBO, catalog no. QPS-201) according to the manufacturer’s protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), \textbeta-actin or HPRT were used to normalize the mRNA. Sequence-specific primers were designed as follows: HSD11\textbeta1, sense 5’-tctctctctgctgaaag-3’, antisense 5’-gaacccatccaagcatacctg-3’; IL-6, sense 5’-gaagcagcaagcactag-3’, antisense 5’-ttcaggcagaggctct-3’; GAPDH, sense 5’-gagttgactggttggctgta-3’, antisense 5’-gacactcatctcctactaccaagtaa-3’; \textbeta-actin, sense 5’-ggcatctcaccctgaagta-3’, antisense 5’-ggctgttgtaaggttccaa-3’; and HPRT, sense 5’-aatgctgctgtaagaga-3’, antisense 5’-aagcagatgaccagact-3’. The qRT-PCR (40 cycles of denaturation at 92°C for 15 sec and annealing at 60°C for 60 sec) was performed on a ViiA7 PCR system (Applied Biosystems).

**Selective 11\textbeta-HSD1 inhibitor treatment**

The 11\textbeta-HSD1 inhibitor PF915275 (Tocris Bioscience, catalog no.3291) is a potent inhibitor of 11\textbeta-HSD1. The inhibitor was dissolved in DMSO and diluted 10,000-fold in culture medium. DMSO was used as a vehicle control.

**Enzyme-linked immunosorbent assay (ELISA)**

NHEKs (100,000 cells/ml, 500 \mu l/well) were seeded in a 24-well type-1 collagen-coated plate. Cells were allowed to attach for 24 h. Epilife was then changed to basal media, which did not contain cortisol or bovine pituitary extract. Concentrations of IL-6 (catalog no. D6050) and cortisol (catalog no. KGE008B) were measured using Quantikine Immunoassays (R&D Systems). Assays were performed according to the manufacturer’s protocols.

**Small interfering RNA (siRNA) transfection**

NHEKs (100,000 cells/ml) were seeded on type-1 collagen-coated plates 1 day prior to transfection. Cells were transfected with 10 nM HSD11B1 siRNA (Invitrogen) or control siRNA (Invitrogen, catalog no.46-
5371) using RNAiMAX (Invitrogen, catalog no. 56532) according to the manufacturer’s protocol. Culture media was replaced 6 h after transfection. Cells were used for experiments 24 h after transfection.

**MTS cell viability assay**

Cellular viability was assessed using a CellTiter96® Aqueous One Solution Cell Proliferation Assay (Promega, catalog no. G358B). Briefly, NHEKs were seeded into 96-well plates (10,000 cells/well in 100 µl medium). MTS reagent was added in 20 µl, and the cells were incubated for 2 h. Optical density was measured at 490 nm with a microplate reader (Bio-Rad).

**Western blotting**

Proteins (10 µg) were separated on sodium dodecyl sulfide-polyacrylamide (SDS-PAGE) gels and transferred onto polyvinylidene fluoride membranes (Millipore, catalog no. IPVH08100). Non-specific protein binding was blocked by incubating the membranes in 3% w/v non-fat milk powder in TBS-T (TBS-T milk). Next, the membranes were incubated with rabbit anti-11β-HSD1 antibody (1:1000; Gene Tex, catalog no. GTX104626), or mouse monoclonal anti-β-actin (1:5000; Sigma-Aldrich, catalog no. A5441) overnight at 4°C. The membranes were washed three times in TBS-T for 5 min each. Finally, the membranes were incubated with either horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody at a dilution of 1:10,000 for 60 min at room temperature. After washing with TBS-T, protein bands were detected using an ECL Plus kit (Thermo scientific, catalog no. 32132).

**Statistical analysis**

Data are expressed as means ± standard deviation (SD). Analysis of variance (ANOVA) for the groups was performed followed by Bonferroni or Dunnett tests for multiple comparisons to allow pairwise testing for significant differences between groups. The student’s t-test was used to determine the level of significance of differences between the two groups. Statistical significance was defined as P<0.05.

**Results**

**EGF suppressed inflammatory responses in NHEKs**

EGF regulates keratinocyte proliferation and induces epithelial mesenchymal transition in keratinocytes. We examined whether EGF has immunosuppressive effects in keratinocytes. Addition of EGF to NHEK significantly suppressed transcript and protein levels of TNF-α-induced IL-6 (Fig. 1 A, B, P<0.05, N = 3 and P<0.001, N = 4, respectively).

**Overexpression of 11β-HSD1 suppressed inflammatory responses in NHEKs**

11β-HSD1 is the enzyme that activates cortisol in cells. To determine whether 11β-HSD1 suppresses inflammatory reactions in NHEKs in vitro, we overexpressed 11β-HSD1 in NHEKs. The 11β-HSD1 transcript levels were significantly enhanced in NHEKs transfected with 1000 ng/ml plasmid; therefore, we used this dose in further experiments (Fig. 1C). Overexpression of 11β-HSD1 significantly suppressed TNF-α-induced IL-6 transcript levels in NHEKs (Fig. 1D, E, P<0.05, N = 3)

In NHEKs in culture, low-dose cortisol (1–10 nM) exerts a proinflammatory effect, whereas physiological to high-dose cortisol (100 nM-1 µM) has an anti-inflammatory effect (Fig. 1F)

**EGF enhanced the expression of 11β-HSD1**

We evaluated the relationship between 11β-HSD1 expression and EGF in NHEKs because we hypothesized that 11β-HSD1 plays a role in EGF-induced immunosuppression. Addition of EGF significantly induced 11β-HSD1 transcript levels in NHEKs in a dose-dependent manner (Fig. 1G). Activated cortisol levels in cell supernatants were increased following addition of 10 ng/ml of EGF (Fig. 1H, P<0.01, N = 6). Increased cortisol in cell supernatants was decreased by 11β-HSD1 knockdown or treatment with a 11β-HSD1 inhibitor (Fig. 1I, J, P<0.05 and P<0.01 respectively, N = 6).

**Inhibition of 11β-HSD1 reversed EGF-induced immunosuppression in NHEKs**

To determine whether increased 11β-HSD1 expression in response to EGF is associated with EGF-induced immunosuppression in NHEKs, we treated
Figure 1. (For figure legend, see page 5.)
NHEKs with an 11β-HSD1 inhibitor PF915275. Inhibition of 11β-HSD1 by PF915275 was not toxic to NHEKs up to 10 μM; therefore, we used this dose in further experiments (Fig. 2A). TNF-α-induced IL-6 transcript levels were significantly upregulated following treatment with the 11β-HSD1 inhibitor (Fig. 2B, P<0.001, N = 3). In addition, 11β-HSD1 inhibition reversed EGF-induced immunosuppression in NHEKs (Fig. 2B, P<0.001, N = 3). Similarly, ELISAs indicated that IL-6 production by NHEKs was significantly increased following treatment with the 11β-HSD1 inhibitor, and EGF-induced immunosuppression was reversed (Fig. 2C, P<0.001, N = 4).

**Knockdown of 11β-HSD1 reversed EGF-induced immunosuppression in NHEKs**

We knocked down 11β-HSD1 expression using siRNA and evaluated EGF-induced immunosuppression in NHEKs. 11β-HSD1 transcript levels were sufficiently knocked down by siHSD11B1 transfection in NHEKs (Fig. 2D, E). TNF-α-induced IL-6 transcript levels were significantly higher in the 11β-HSD1 knocked down group compared with the negative control siRNA transfected group. In addition, 11β-HSD1 knockdown reversed EGF-induced immunosuppression in NHEKs (Fig. 2F, P<0.001, N = 3). Knockdown of 11β-HSD1 also enhanced TNF-α-induced IL-6 production (P<0.001, N = 4), but its reverse effect on EGF-induced immunosuppression was not apparent when evaluated by ELISA (Fig. 2G).

**Cetuximab decreased the expression of 11β-HSD1 in NHEKs**

Treatment of NHEKs with cetuximab, an EGFR inhibitor, increases IL-6, IL-8, and TNF-α transcript levels in a dose-dependent manner. Similarly, in our study, cetuximab treatment of NHEKs increased levels of both IL-6 transcript and protein in both untreated and TNF-α-treated cells (Fig. 3A, B). We evaluated whether EGFR inhibition by cetuximab affects the expression of 11β-HSD1 and found that it decreased 11β-HSD1 transcript levels in NHEKs (Fig. 3C, P<0.01, N = 3) and the decrease was more apparent in TNF-α-treated group (P<0.001, N = 3). However, the changes in the levels of cortisol in the supernatants were not apparent (Fig. 3D).

**Discussion**

Our results suggest that a mechanism of the immunosuppressive effect of EGFR signalling is through the activation of local cortisol by 11β-HSD1 in keratinocytes. Although EGFR signalling has been reported to have *in vivo* immune-modulatory effects in skin, there are no reports specifically examining keratinocytes *in vitro*. Thus, we investigated whether EGFR signalling has immune-modulatory effects in keratinocytes.
Figure 2. 11β-HSD1 inhibition or knockdown reversed EGF-induced immunosuppression in NHEK (A) NHEKs treated with an 11β-HSD1 selective inhibitor (0, 1, 5, or 10 μM) for 48 h were assessed by a MTS assay. DMSO was used as a control (N = 7 per group). (B and C) qRT-PCR (B) and ELISA (C) analysis of IL-6 in NHEKs treated with an 11β-HSD1 inhibitor. NHEKs were treated with an 11β-HSD1 inhibitor (10 μM) and/or EGF (10 ng/ml) with or without TNF-α (10 ng/ml). TNF-α was added 1 day after EGF addition. Cortisone (1 μM) was added 10 minutes before TNF-α addition in all samples and an 11β-HSD1 inhibitor was added 10 minutes prior to cortisone in indicated samples. Cells were harvested 4 hours after TNF-α treatment for qRT-PCR and supernatant were harvested 24 hours after TNF-α treatment for ELISA. GAPDH was used as an internal control [N = 3 (qRT-PCR), N = 4 (ELISA); **P<0.01, ***P<0.001 as assessed by Student’s t-test]. (D) qRT-PCR analysis of HSD11B1 in NHEKs transfected with control siRNA or HSD11B1 siRNA for 48 hours. A representative western blot of 11β-HSD1 relative to the β-actin loading control is shown (N = 3). (E, F and G) qRT-PCR (E and F) and ELISA (G) analysis of IL-6 and HSD11B1 in NHEKs transfected with control siRNA or HSD11B1 siRNA. TNF-α was added 1 day after EGF addition. Cortisone (1 μM) was added 10 minutes before TNF-α addition in all samples and cells were harvested 4 hours after TNF-α treatment for qRT-PCR and supernatant were harvested 24 hours after TNF-α treatment for ELISA. GAPDH was used as an internal control [N = 3 (qRT-PCR), N = 4 (ELISA); **P<0.01, ***P<0.001 as assessed by Student’s t-test].
and found that it does have strong immunosuppressive effects. As physiological doses of cortisol (100 nM-1 μM) exert anti-inflammatory effects (Fig. 1F), activation of cortisol through 11β-HSD1 is thought to also exert anti-inflammatory effects.

Because local cortisol activation by keratinocytes via 11β-HSD1 also has immunosuppressive effects and because EGF induced the expression of 11β-HSD1 in keratinocytes, we hypothesized that EGF-induced immunosuppression in keratinocytes may function via induction of local cortisol activation. Thus, we measured the concentrations of cortisol in the supernatants of NHEKs following treatment with EGF. Compared with a significant increase of 11β-HSD1 transcript, the increase in supernatant cortisol was relatively mild (Fig. 1G). We suspect that this difference is because supernatant cortisol concentrations in keratinocytes reflect cortisol produced by both the de novo pathway through CYP11A1 and CYP11B1 and the activating pathway through 11β-HSD1. In addition, most of the cortisol produced in cells binds to glucocorticoid receptors and only minimal amounts of cortisol are released into the supernatant. Thus, the observed increase of cortisol in the supernatants is limited compared with the increase of 11β-HSD1. Next, we confirmed our hypothesis that 11β-HSD1 inhibitor or siRNA inhibition reversed the immunosuppressive effect of EGF (Fig. 2B, C, F). This effect was not apparent at the protein level in the siRNA
Inhibition experiment, possibly due to only transient knockdown of 11β-HSD1 by siRNA. The possibility was supported by the observation that the protein samples were corrected 24 h after the mRNA samples, suggesting that the knockdown by siRNA may have weakened by this point.

We further examined whether local cortisol activation may be involved in EGFR inhibitor-induced adverse effects. EGFR inhibitors are used to treat a variety of tumour types, such as non-small cell lung cancer and squamous cell carcinoma of the head and neck. Papulopustular (acneiform) eruptions are commonly observed as an adverse effect of EGFR inhibitor treatment with an incidence rate of 55%.

We found that EGFR inhibitor increased IL-6 production and decreased the expression of 11β-HSD1 in keratinocytes (Fig. 3A-C). The results were more apparent in TNF-α-treated cells; however, decreases in supernatant cortisol levels were not as intense as those of 11β-HSD1 transcript levels. We surmise that local cortisol activation may, in part, be responsible for the EGFR inhibitor-induced adverse effects, and the effectiveness of topical corticosteroid treatment for papulopustular eruptions supports our interpretation. We also need to consider other possibilities besides local cortisol production in the mechanism of the proinflammatory effects of EGFR inhibitor in keratinocytes because in vitro treatment of NHEKs with an EGFR inhibitor induces cell differentiation, apoptosis, and inflammation. In addition, treatment with an EGFR inhibitor elevates the production of various inflammatory cytokines, such as IL-6, IL-8, and TNF-α, in immortalized human SZ95 sebocytes. We have not evaluated sebocytes in this study but 11β-HSD1 in sebocytes may also be involved in EGFR inhibitor induced skin reaction.

Our results suggest that attenuated local cortisol activation due to decreased expression of 11β-HSD1 by an EGFR inhibitor may be associated with EGFR inhibitor-induced inflammation and skin reactions. In addition to topical corticosteroid, targeting 11β-HSD1 could have potential as a treatment of EGFR inhibitor treatment-induced skin reaction.

**Abbreviations**

ANOVA Analysis of variance
EGFR epidermal growth factor receptor
ELISA Enzyme-linked immunosorbent assay
GAPDH Glyceraldehyde-3-phosphate dehydrogenase
IL-6 interleukin-6
NHEK Normal epidermal human keratinocytes
siRNA Small interfering RNA
SD standard deviation
SDS-PAGE sodium dodecyl sulfide-polyacrylamide
tNF-α tumour necrosis factor α
qRT-PCR quantitative reverse transcriptase-polymerase chain reaction
11-DHC 11-dehydrocorticosterone
11β-HSD1 11β-hydroxysteroid dehydrogenase 1

**Disclosure of potential conflict of interest**

No potential conflicts of interest were disclosed.

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