Aryl Hydrocarbon Receptor Activation Induced by Epidermal Growth Factor Receptor Inhibitors in Human Keratinocytes and Sebocytes: A Possible Mechanism of Acneiform Eruption

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Aryl Hydrocarbon Receptor Activation Induced by Epidermal Growth Factor Receptor Inhibitors in Human Keratinocytes and Sebocytes : A Possible Mechanism of Acneiform Eruption

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Abstract

Although epidermal growth factor receptor inhibitors (EGFRIs) are effective in treating advanced carcinomas, they frequently cause acneiform eruption, which could be related to sebaceous gland activity ; however, the mechanism remains largely unknown. Based on the facts that 1) activation of aryl hydrocarbon receptor (AHR) regulates follicular and epidermal keratinocyte differentiation and sebaceous gland activity, 2) EGFR signaling compensates for AHR signaling by sharing p300, and 3) sebum production is increased during EGFRI treatment, we hypothesized that EGFRI-induced activation of AHR may be involved in the development of acneiform eruption.

To demonstrate this, we administered clinically utilized EGFRIs, namely gefitinib and anti-EGFR antibody (Ab), to normal human epidermal keratinocytes (NHEKs) and human sebocyte cell line SEB-1, and examined whether gefitinib and anti-EGFR Ab induced AHR activation. Gefitinib treatment induced up-regulation of CYP1A1 mRNA in NHEKs and SEB-1 cells, which was further enhanced by 6-formylindolo (3,2-b) carbazole (FICZ), an endogenous AHR ligand. Although anti-EGFR antibody treatment alone did not induce up-regulation of CYP1A1 mRNA in NHEKs and SEB-1 cells, it enhanced FICZ-induced up-regulation of CYP1A1 mRNA. In the SEB-1 cells, sebum production was increased by gefitinib treatment, and further enhanced by FICZ. Moreover, knockdown of AHR expression, using siRNA transfection of AHR, inhibited sebum production by gefitinib treatment, thereby indicating that EGFRI-induced AHR activation is involved in the increase of sebum production.

To extend our in-vitro observation, we orally administered gefitinib to C57BL/6 mice and applied FICZ on their abdomen. Gefitinib treatment induced up-regulation of CYP1A1 mRNA in the epidermis, which was enhanced by topical application of FICZ. These data together suggest that AHR activation in keratinocytes and sebocytes, with subsequent sebum production induced by EGFRIs, may play an important role in the development of acneiform eruption.

Keywords : AHR, EGFR inhibitor, acneiform eruption

Introduction

Although epidermal growth factor receptor inhibitors (EGFRIs) are effective in treating advanced carcinomas, EGFRIs often cause adverse effects on the skin, most commonly

Abbreviation :
AHR : Aryl hydrocarbon receptor
EGF : Epidermal growth factor
EGFR : Epidermal growth factor receptor
FICZ : 6-formylindolo (3, 2-b) carbazole

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acneiform eruption, a papulopustular reaction on the skin[1] ; it requires therapeutic intervention in approximately one third of the patients[2].

Although the skin rash usually resolves after interrupting the EGFRI treatment, the condition affects both optimal efficacy of the treatment and quality of life negatively. In addition, the severe skin rash may lead to dose-modification or treatment discontinuation, thereby resulting in reduced clinical benefits from the EGFRI treatment. Importantly, several retrospective studies on the relationship between EGFRI treatment and adverse skin reactions have revealed the appearance and severity of EGFRI treatment-related skin rash to be positively correlated with tumor response to EGFRI and subsequently good prognosis[3)~5)].

These observations together support the consensus that acneiform eruption should be treated while continuing EGFRI treatment, in order to derive the maximal clinical benefit from EGFRI treatment against advanced carcinomas.

Several studies on the pathomechanism of acneiform eruption, caused by EGFRI treatment, have shown EGFR inhibition to modify chemokine and inflammatory cytokine production[6], anti-bacterial peptide production[7], and follicular and epidermal keratinocyte differentiation[8], thereby contributing to the development of acneiform eruption.

Since EGFR signaling has been shown to compensate for aryl hydrocarbon receptor (AHR) signaling by sharing p300[9] (a transcriptional factor), we hypothesized that the inhibition of EGFR by EGFRI treatment would cause acneiform eruption via activation of AHR. To the best of our knowledge, there are only a few studies that have investigated the role of AHR in the development of EGFRI treatment-induced acneiform eruption.

AHR, a ligand-activated transcription factor, is preferentially expressed in the epidermis, and regulates keratinocyte differentiation[10]. Although dioxins such as TCDD were identified as the ligands of AHR, recent studies have revealed FICZ, a photo-product derived from tryptophan, as an endogenous ligand[11]. The ligand-activated AHR induces its nuclear translocation from the cytoplasm, thereby leading to the up-regulation of its target genes such as CYP1A1 in normal human keratinocytes (NHEKs)[12].

AHR activation is reportedly crucial for the development of chloracne, a specific acneiform eruption observed in patients with TCDD-poisoning. TCDD induces the up-regulation of SLPI, SPPR2D, and EPGN via AHR activation, leading to acanthosis and hyperkeratosis of the infundibular keratinocytes[13]. Therefore, we examined whether AHR activation, induced by EGFRI treatment in NHEKs, is involved in the development of acneiform eruption.

Our clinical research has revealed that sebum production is increased, since the initiation of EGFRI treatment[14], thereby suggesting stimulation of sebaceous gland activity by EGFR inhibition ; however, the precise mechanism remains largely unknown.

We also examined how the activation of AHR, induced by EGFRI treatment, could modify sebum production by sebaceous glands, using immortalized human sebocyte cell line SEB-1.

In the current study, we administrated clinically utilized EGFRIs, namely gefitinib and anti-EGFR antibody (Ab), to NHEKs and SEB-1 cells and examined whether they induced AHR activation. To extend our in-vitro observation, we also attempted to utilize an in-vivo model. We orally administered gefitinib to C57BL/6 mice and examined whether it could enhance the AHR activation induced by topical application of FICZ.

Results

To examine whether EGFRI treatment activates AHR signaling, we evaluated mRNA expression of CYP1A1, a representative AHR downstream gene, in gefitinib- or anti-EGFR Ab-treated NHEKs and SEB-1 cells. Gefitinib treatment induced up-regulation of CYP1A1 mRNA in

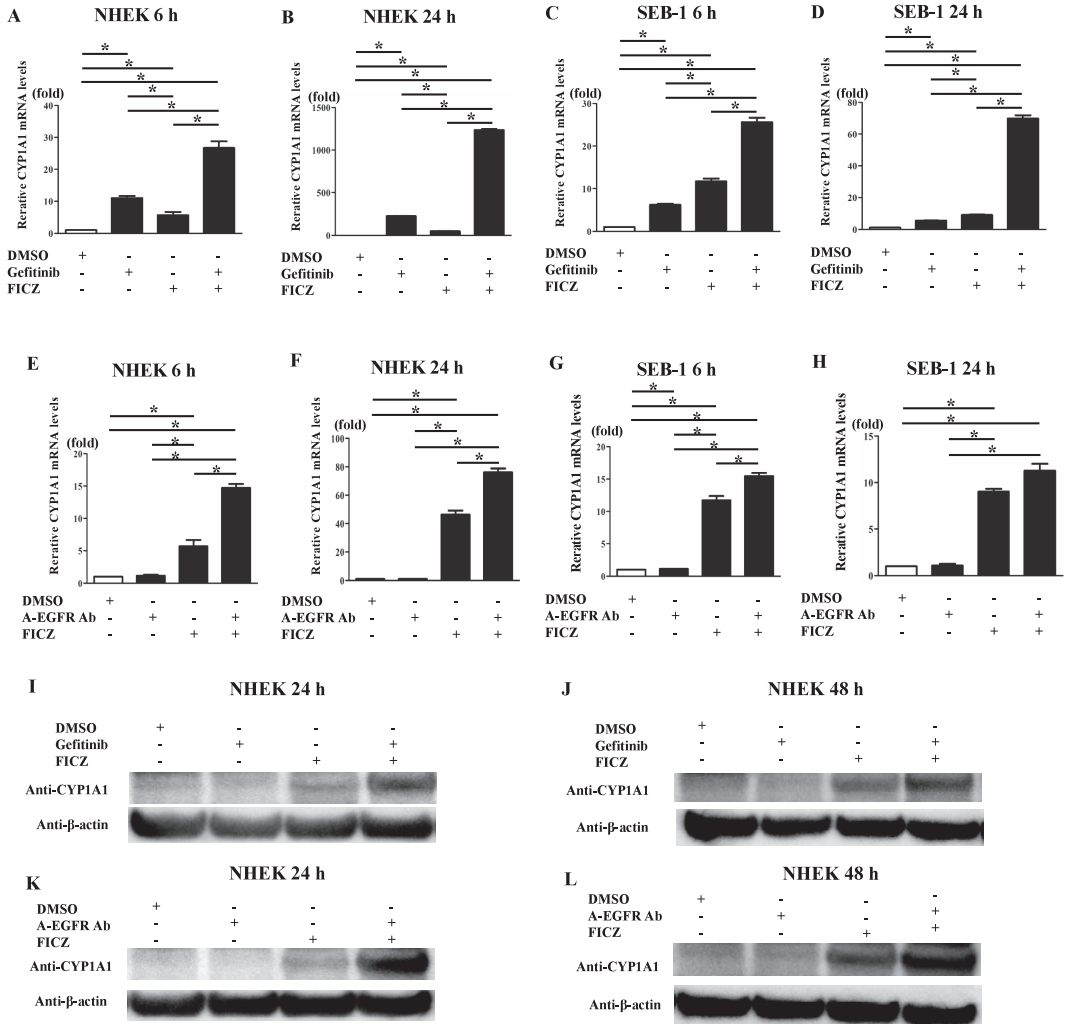

Fig 1. Either gefitinib or anti-EGFR Ab treatment enhanced the up-regulation of CYP1A1, induced by FICZ treatment, in NHEKs and SEB-1 cells.

(**A–H**) NHEKs and SEB-1 cells were treated with FICZ (100 nM) in presence or absence of either gefitinib (1.25 μg/ml) or anti-EGFR Ab (0.5 μg/ml) for indicated time periods. CYP1A1 mRNA expression was analyzed by qRT-PCR. Data are expressed as mean ± S. E. M. ; n = 3 for each group. Statistical differences between the expression of control and treated NHEKs are presented ; $^{*}P < 0.05$. (I, J, K, and L) CYP1A1 protein expression was analyzed with an anti-CYP1A1 antibody using western blotting. The data are representative of experiments repeated thrice with similar results.

NHEKs (Fig. 1A and 1B) and SEB-1 cells (Fig. 1C and 1D). Anti-EGFR Ab treatment alone did not induce up-regulation of CYP1A1 mRNA in either NHEKs (Fig. 1E and 1F) or SEB-1 cells (Fig. 1G and 1H) ; however, they enhanced FICZ treatment-induced up-regulation of CYP1A1 mRNA (Fig. 1A–H). From western blot analysis, we observed up-regulation of CYP1A1 protein in NHEKs (Fig. 1I, 1J, 1K, and 1L), but not in SEB-1 cells (data not shown).

Our previous clinical research on acneiform eruption, during EGFRI treatment, had shown sebum production of the skin to be subsequently enhanced after the initiation of EGFRI treatment[14]. Therefore, we hypothesized that AHR activation, induced by EGFRI treatment, is involved in the increase of sebum production. To test this, we examined whether EGFRI treatment increased sebum production, which was enhanced by FICZ treatment in SEB-1 cells. Sebum production in the cytoplasm of SEB-1 cells was evaluated using BODIPY staining. Gefitinib treat-

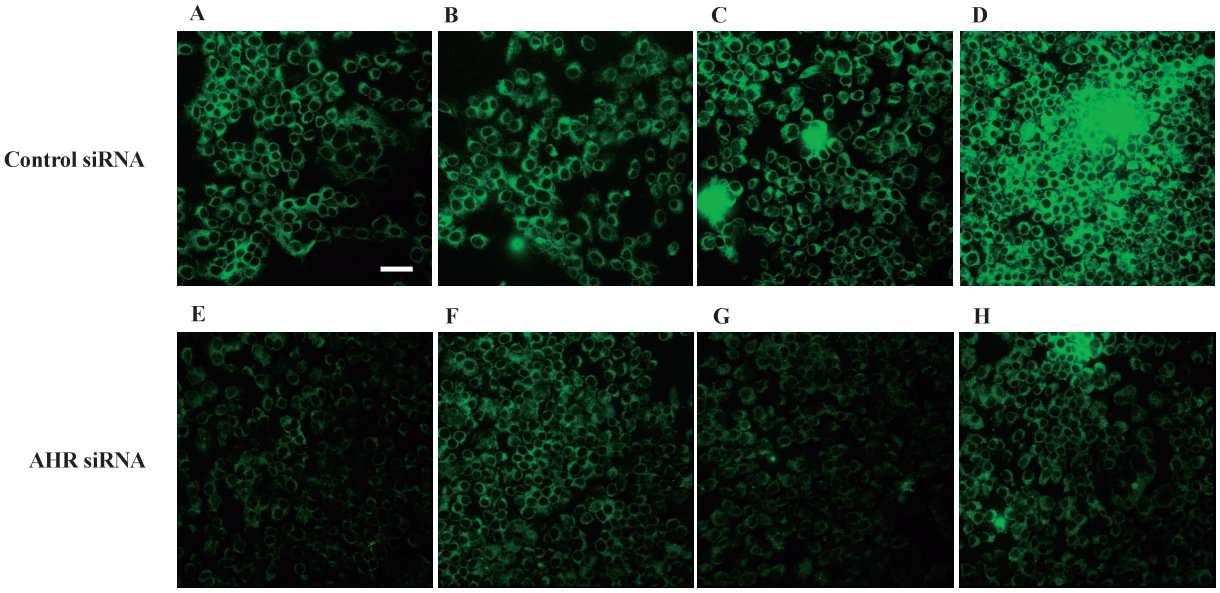

Fig 2. Gefitinib treatment enhanced the sebum production induced by FICZ treatment in SEB-1 cells, which was dependent on AHR.

(**A**–**H**) Either control siRNA- or AHR siRNA-transfected SEB-1 cells were treated with FICZ (100 nM) in presence of gefitinib (1.25 μg/ml) for 24 h. Sebum production was evaluated by BODIPY staining (Green). (A and E) control ; (B and F) Gefitinib ; (C and G) FICZ ; (D and H) Gefitinib plus FICZ. Data are representative of experiments repeated thrice with similar results. The scale bar (in A) is 25 μm.

ment increased sebum production, which was further enhanced by FICZ treatment (Fig. 2C and 2D). To determine whether the increase of sebum production is dependent on AHR, we knocked down the expression of AHR (siRNA AHR) using siRNA transfection. Knockdown of AHR inhibited the sebum production induced by gefitinib-plus-FICZ treatment (Fig. 2H), thus indicating that EGFRI treatment increased sebum production via AHR activation in SEB-1 cells. Knockdown of AHR was previously confirmed by qRT-PCR analysis[12].

To examine whether EGFRI treatment induces AHR activation in vivo, we administered gefitinib to C57BL/6 mice and applied FICZ topically on the abdomen, as described in a previous report[15]. qRT-PCR analysis of the abdomen skin revealed the induced up-regulation of CYP1A1 mRNA levels by topical application of FICZ, which was enhanced by gefitinib treatment (Fig. 3), implying a possibility that gefitinib enhances AHR activation in vivo.

Discussion

In the current study, we have shown for the first time that EGFRI treatment induces AHR activation in human keratinocytes and sebocytes. Our results suggest that the EGFRI treatment-induced AHR activation in NHEKs may lead to follicular and epidermal keratinocyte differentiation, contributing to the development of acneiform eruption. Considering the fact that FICZ is a photo-product generated from tryptophan[16], ultra violet irradiation such as sunlight-exposure might possibly worsen the acneiform eruption caused by EGFRI ; hence, proper protection from sun light may be useful during the treatment of acneiform eruption.

Our clinical research on sebum production in EGFRI treatment-induced acneiform eruption has shown EGFRI treatment to increase sebum production[14], which is consistent with the finding of EGFRI inducing sebum production in SEB-1 cells. In addition, FICZ treatment enhanced EGFRI-induced sebum production, suggesting

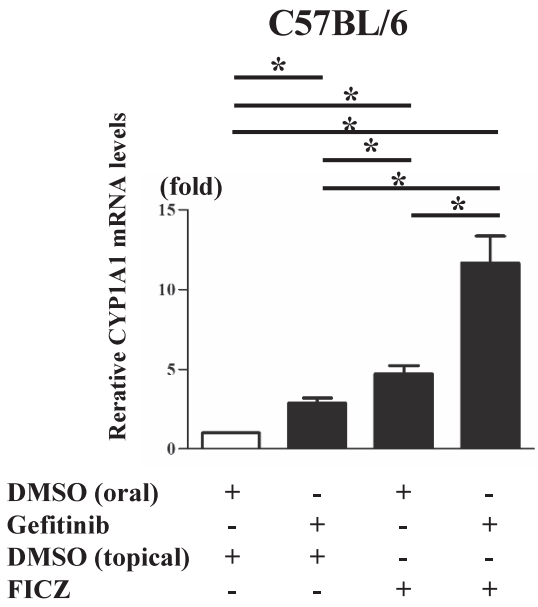

Fig. 3 Gefitinib treatment enhanced the up-regulation of CYP1A1 mRNA in the epidermis induced by topical application of FICZ in vivo.

Gefitinib (50 mg/kg) was administered to C57BL/6 mice by oral gavage (200 μl per mouse). On day 0, the abdomen of each mouse was shaved and DMSO or gefitinib was administered. On day 1, 1 μM of FICZ, dissolved in DMSO, was applied to the abdomen topically (20 μl/mouse) ; 6 h later, the abdomen skin was collected to analyze mRNA levels, using qRT-PCR. The epidermis was separated from the dermis and mRNA was extracted from the epidermis. Data are expressed as mean ± S. E. M. ; n = 5 for each group. Statistical differences between the expression in control and treated-groups are presented ; $^*P < 0.05$.

that AHR activation by FICZ is one of the triggers toward the development of acneiform eruption. These data suggest that antagonists against AHR, such as CH223191, may be promising agents in the treatment of EGFRI-induced acneiform eruption in SEB-1 cells. The mechanism by which AHR activation increases sebum production is not yet clear. Considering the facts that PPAR-γ expression is a key determinant of sebum production in sebaceous glands[17], EGFRI treatment up-regulates PPAR-γ expression[18], and TCDD treatment increases adipocyte differentiation along with PPAR-γ up-regulation[19], there is a possibility that up-regulation of PPAR-γ via AHR activation may mediate EGFRI treatment-induced sebum production ; however, further studies would be required to reveal the vital relationship between AHR and PPAR-γ in sebum production.

Finally, in-vivo study using gefitinib showed that gefitinib treatment enhances FICZ-induced up-regulation of CYP1A1 mRNA, thereby suggesting a possibility that EGFRI treatment activates the AHR-Nrf2 axis in the epidermis in vivo. Taken together, we have demonstrated that activation of AHR, induced by EGFRI treatment, in human keratinocytes and sebocytes, may partially explain the mechanism by which EGFRI treatment results in acneiform eruption.

Materials & Methods

Reagents and antibodies

Gefitinib from Cayman Chemical (Ann Arbor, MI) and FICZ from Enzo Life Sciences (Exeter, United Kingdom) were dissolved in DMSO (St. Louis, MO) and stored at −30℃ until further use. Anti-EGFR-Ab was purchased from Merck Millipore (Darmstadt, Germany).

Anti-human CYP1A1 mouse monoclonal IgG antibody (ab79819) from Abcam (Cambridge, UK) and anti-human β-actin mouse monoclonal IgG antibody (#3700) from Cell Signaling Technology (Danvers, MA) were utilized in western blot analysis.

Cell culture and treatments

NHEKs, obtained from Clonetics-BioWhittaker (San Diego, CA), were grown in culture dishes at 37℃ in 5% CO_2. They were cultured in serum-free KC growth medium (Lonza, Walkersville, MD) supplemented with bovine pituitary extract, recombinant epidermal growth factor, insulin, hydrocortisone, transferrin, and epinephrine. Culture medium was replaced every 2 days.

SEB-1 cells, kindly gifted by Dr. Diane Thiboutot[20], were grown to confluence in all experiments and cultured in Dulbecco's Modified

Eagle's Medium (DMEM) supplemented with 5.5 mM glucose (Sigma-Aldrich, St. Louis, MO)/ Ham's F-12 (Sigma-Aldrich) in 3 : 1 ratio, 2.5% fetal bovine serum (Japan Bioserum Co. Ltd., Hiroshima, Japan), 1.8×10^{-4} M adenine (Sigma-Aldrich), 0.4μg/ml hydrocortisone (Sigma-Aldrich), 10 ng/m ; insulin (Sigma-Aldrich), 3 ng/ml epidermal growth factor (PeproTech, Rocky Hill, NJ), and 1.2 ×10-10 M cholera toxin (Wako Pure Chemical Industries Ltd., Osaka, Japan).

NHEKs or SEB-1 cells (1×10^5) were seeded in 24-well culture plates, allowed to attach for 24 h, and subsequently treated with or without DMSO, gefitinib, anti-EGFR-Ab, and FICZ.

Confocal laser scanning microscopy of sebum production using BODIPY staining

SEB-1 cells (2.5×10^5), cultured on 35-mm dish, were treated with or without DMSO, gefitinib, anti-EGFR-Ab, and FICZ for 24 h, and washed in phosphate-buffered saline. Sebum staining using BODIPY (Cosmo Bio Co., Ltd., Tokyo, Japan) was performed according to the manufacture's protocol (Adipocyte Fluorescent Staining Kit). All samples were analyzed using a D-Eclipse laser scanning confocal microscope (Nikon, Tokyo, Japan).

Quantitative reverse transcription (qRT)-PCR analysis

Total RNA was extracted using the RNeasy® Mini kit (Qiagen, Courtaboeuf, France). Reverse transcription was performed using PrimeScript™ RT reagent kit (TaKaRa Bio, Otsu, Japan). qRT-PCR was performed on a CFX Connect™ Real-time System (Bio-Rad, Hercules, CA) using SYBR® Premix Ex Taq (TaKaRa Bio). Amplification was initiated at 95℃ for 30 s, followed by 40 cycles of qRT-PCR at 95℃ for 5 s and 60℃ for 20 s. mRNA expression was measured in triplicate and normalized to that of β-actin. Primer sequences from TaKaRa Bio and SABiosciences (Frederick, MD) are shown below :
β-actin :
Forward 5'-ATTGCCGACAGGATGCAGA-3' ;
Reverse 5'-GAGTACTTGCGCTCAGGAGGA-3'
The sequence of the CYP1A1 primers (PPH 01271E) was not opened.

Western blotting

Cells were incubated in lysis buffer for 5 min (Complete lysis M ; Roche Diagnostics, Basel, Switzerland). Protein concentration of the lysate was measured using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Equal amounts of protein (20 μg) were dissolved in NuPage LDS sample buffer (Invitrogen) in presence of 10% sample reducing agent (Invitrogen). The lysates were boiled at 70℃ for 10 min and then loaded and run on NuPage 4 % ～12% Bis-Tris gels (Invitrogen) at 200 V for 60 min. The proteins were then transferred on to polyvinylidene difluoride membranes (Invitrogen) and blocked in Western-Breeze Blocker/Diluent (Invitrogen). The membranes were then probed with anti-CYP1A1 and anti-β-actin antibody overnight at 4℃. Anti-mouse horseradish peroxidase-conjugated IgG antibody (Cell signaling) was used as a secondary antibody. Visualization of protein bands was accomplished using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) by ChemiDoc Touch Imaging System (Bio-Rad).

Transfection of siRNAs against AHR

siRNAs against AHR (AHR siRNA, s1200), as well as those with a scrambled sequence that would not lead to specific degradation of any cellular message (control siRNA), were purchased from Ambion (Austin, TX). NHEKs or SEB-1 cells, cultured in 24-well plates, were incubated for 48 h in 0.5 ml of culture medium, along with 5 nM siRNA and 3 μl of HiPerFect Transfection Reagent (Qiagen).

Animal experiment procedure

C57BL/6 mice were obtained from the Kyushu University Institute's Animal Production Program (Fukuoka, Japan), housed in a clean facility,

and bred and used in accordance with institutional guidelines. A total of 10 mg of gefitinib was first dissolved in 160 μl of DMSO and diluted with 1440 μl of sterile olive oil. Gefitinib was administered to mice by means of oral gavage (200 μl/mouse). On day 0, the abdomen of each mouse was shaved and DMSO or gefitinib was administered to the mice. On day 1, 1 μM of FICZ, dissolved in DMSO, was topically applied on the abdomen (20 μl/mouse). 6h later, skin sample was collected to analyze mRNA levels using qRT-PCR.

The skin was floated with dermal side down and incubated with 3.8% ammonium thiocyanate for 30 min. The epidermis was separated from the dermis and mRNA extraction from the epidermis was performed immediately after, using TRIzol (Invitrogen), according to the manufacture's protocol.

Statistical analysis

Unpaired Student's t-test or one-way analysis of variance was used to analyze the results. A p-value of <0.05 was considered to indicate a statistically significant difference. All data are presented as mean ± S. E. M. ; n = 3 for three independent experiments.

Conflict of interest

The authors state no conflict of interest.

Acknowledgments

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（和文抄録）

上皮成長因子受容体阻害薬によって生じる芳香族炭化水素受容体の活性化について：ざ瘡様発疹症の発症機序の可能性

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上皮成長因子受容体阻害薬は進行期の癌に対して有効であるが，しばしば，副作用としてざ瘡様発疹症を生じる．これには脂腺の活動性が増加することが関連することが示唆されているが，その機序はほとんど知られていない．我々は，1）芳香族炭化水素受容体が毛包上皮細胞や表皮細胞の細胞分化を制御し，脂腺の活動性も制御すること，2）上皮成長因子受容体シグナルと芳香族炭化水素受容体シグナルは p300 タンパクを共有しており，お互いに競合しあうこと，3）上皮成長因子受容体阻害薬の投与を開始すると皮脂の分泌が増加すること，という事実に基づき，上皮成長因子受容体阻害薬によって芳香族炭化水素受容体の活性化されることが，ざ瘡様発疹症の発症に関与しているのではないかという仮説を立てた．

この仮説を証明するために，正常ヒト表皮細胞とヒト脂腺細胞の cell line である SEB-1 細胞に対して，上皮成長因子受容体阻害薬（ゲフィチニブ，抗上皮成長因子受容体抗体）を投与し，芳香族炭化水素受容体が活性化されるかを検討した．ゲフィチニブ，抗上皮成長因子受容体抗体の投与は，CYP1A1 の発現の増加を誘導し，この現象は芳香族炭化水素受容体に対する内因性リガンドである FICZ を投与するとさらに増強した．また，SEB-1 細胞にゲフィチニブを投与したところ，皮脂の分泌が増加し，FICZ を投与するとさらに皮脂の分泌は増加した．さらに，芳香族炭化水素受容体の発現を siRNA によって低下させた状態では，ゲフィチニブを投与しても皮脂の分泌は増加しなかった．

さらに，in vivo でも同様の結果が得られるか確かめるため，C57BL/6 マウスに対してゲフィチニブを経口投与し，同時に腹部に FICZ を外用した．腹部の皮膚を回収し，CYP1A1 の発現について解析したところ，ゲフィチニブを投与すると，CYP1A1 の発現の増加が誘導され，FICZ を外用するとさらに CYP1A1 の発現の増加が増強された．

以上から，上皮成長因子受容体阻害薬によって生じる芳香族炭化水素受容体の活性化は，ざ瘡様発疹症の発症において重要な役割をしている可能性が示唆された．

キーワード：芳香族炭化水素受容体，上皮成長因子受容体阻害薬，ざ瘡様発疹症