



Epidermal growth factor receptor inhibitors selectively inhibit the expressions of human β -defensins induced by *Staphylococcus epidermidis*



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ABSTRACT

Background: Epidermal growth factor receptor inhibitors (EGFRIs) have developed as one of the potential treatment options for various kinds of cancers. Although a variety of dermatological adverse reactions such as follicular acneiform eruptions is commonly encountered, the mechanism of the reactions remains unclear.

Objectives: We investigated the effects of EGFRIs on the expression of human β -defensins against staphylococci to study the pathomechanism of cutaneous adverse reactions caused by EGFRIs.

Methods: We investigated the expressions of human β -defensins 1, 2, and 3 (hBD1, 2, and 3) from staphylococci-stimulated normal human epidermal keratinocytes (NHEKs) cultured with or without the effects of two EGFRIs, gefitinib and erlotinib. We stimulated NHEKs with the supernatant of *Staphylococcus aureus* (*S. aureus*) and *S. epidermidis* and the live staphylococci. We measured hBDs in the culture supernatants of NHEKs by enzyme-linked immunosorbent assay (ELISA).

Results: EGFRIs did not suppress the expressions of hBD1 and 3 induced by *S. aureus*. In contrast, EGFRIs suppressed the expressions of hBD2 and 3 induced by *S. epidermidis*.

Conclusion: EGFRIs may cause cutaneous adverse effects through selectively perturbing innate immune responses induced by commensal and pathogenic bacteria.

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1. Introduction

The use of epidermal growth factor receptor inhibitors (EGFRIs) is well established as a therapy for various solid tumors. However, a considerable number of patients have developed cutaneous adverse effects to this treatment, such as follicular acneiform eruptions, xerosis and chronic paronychia. These dermatologic toxicities can lead to in compliance and dose reduction or even cessation of anti-EGFR therapy and have been shown to compromise patients' quality of life.

Activation of EGFRs has been shown to regulate normal keratinocyte proliferation, migration and survival; therefore, inhibition of the EGFR-mediated signalling pathways affects keratinocytes by inducing growth arrest and apoptosis, decreasing cell migration, increasing cell attachment and differentiation, and stimulating inflammation, all of which result in distinctive cutaneous manifestations [1]. However, the precise mechanism by which EGFRIs induce adverse cutaneous effects is still unknown. Keratinocytes actively participate in the innate immune response through the production of cytokines, chemokines [2] and microbial peptides [3]. In human skin, antimicrobial peptides such as human β -defensins (hBDs) serve as the first line of defense against infection by pathogenic microorganisms, being active against a broad spectrum of Gram-positive and Gram-negative bacteria, as well as some fungi and enveloped viruses [4].

In this study, we analysed how EGFRIs affect the innate immune response in keratinocytes upon contact with the

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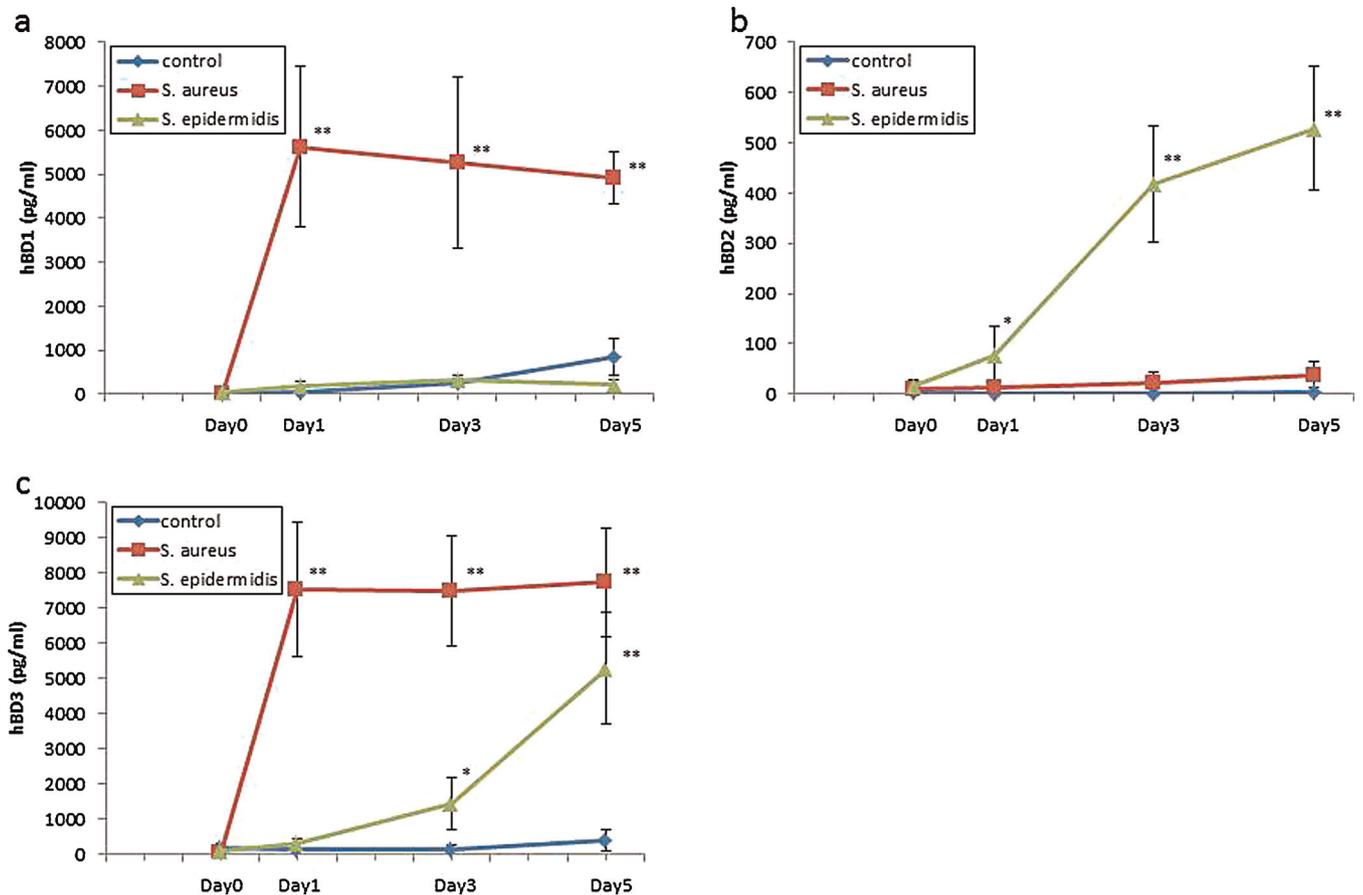


Fig. 1. Expression levels of hBDs induced by staphylococci. The expression levels of hBD1 (a), hBD2 (b) and hBD3 (c) in the culture supernatants of NHEKs stimulated with the staphylococcal supernatants were evaluated by ELISA. Data represent the means \pm standard deviations from three experiments. *P* values were evaluated using Student's *t*-test (control vs. *S. aureus* or *S. epidermidis*, **p* < 0.05; ***p* < 0.01).

pathogenic *Staphylococcus aureus* (*S. aureus*) and the commensal *S. epidermidis*.

2. Materials and methods

2.1. The preparation of bacteria and the culture supernatants

S. aureus: IID980 and JCM2151, and *S. epidermidis*: ATCC12,228 and JCM2414 were used for the experiment. Clinical isolates of *S. aureus* and *S. epidermidis* were confirmed by 16S rRNA sequences as previously described [5]. Other staphylococcal strains (*S. aureus*: JCM2151 and *S. epidermidis*: JCM2414) were provided by RIKEN BRC (Saitama, Japan) through the National Bio-Resource Project of MEXT, Japan. *S. aureus* and *S. epidermidis* were stored at -70°C on Microbank beads (Pro-lab Diagnostics, Ontario, Canada) and grown on tryptic soy agar plates (TSA; Becton, Dickinson and Company, NJ, USA). The colonies observed on TSA plate were picked and inoculated into 10 ml of tryptic soy broth (TSB; Becton, Dickinson and Company, NJ, USA) for an overnight shaking cultivation at 37°C . These bacterial broths were centrifuged at 3000 rpm for 10 min, and the bacterial pellets were washed twice in phosphate-buffered saline (PBS). These bacterial pellets were re-suspended to 10^8 colony forming unit (CFU)/ml with serum-free keratinocyte growth medium for the following stimulation experiments. The bacterial density was confirmed by optical density at 600 nm and counts of CFU. Bacterial supernatants were also prepared to examine the influence of substances secreted by bacteria on keratinocytes. *S. aureus* and *S. epidermidis* were

cultivated in 10 ml of TSB for 3 days at 37°C with shaking. The bacterial density of 3 days culture broths were approximately 10^9 CFU/ml. The bacterial culture supernatants were filtrated using a $0.22\ \mu\text{m}$ Millex-GP filter unit (Millipore, Billerica, MA, USA) and stored at -30°C until use.

2.2. Epidermal growth factor receptor inhibitors preparation

We prepared two kinds of epidermal growth factor receptor inhibitors, gefitinib and erlotinib. They were provided by SYMANSIS NZ Ltd (Washdyke, NZ). We adjusted the final concentration of these drugs at the maximum concentrations of each drug in the human blood (1.25 $\mu\text{g}/\text{ml}$ of gefitinib and 5 $\mu\text{g}/\text{ml}$ of erlotinib). In addition, we used dimethylsulfoxide (DMSO) to dissolve the drugs at the final concentration of 0.01%.

2.3. Keratinocyte culture and stimulations

Normal human epidermal keratinocytes (NHEKs) purchased from Kurabo Industries (Osaka, Japan) were cultured in serum-free keratinocyte growth medium, HuMedia-KG2 (Kurabo Industries, Osaka, Japan), containing human epidermal growth factor (0.1 ng/ml), insulin (10 $\mu\text{g}/\text{ml}$), hydrocortisone (0.5 $\mu\text{g}/\text{ml}$), gentamycin (50 $\mu\text{g}/\text{ml}$), amphotericin B (50 ng/ml) and bovine brain pituitary extract (0.4% v/v) at 37°C in 5% CO_2 . NHEKs at passage three or four were seeded to 6 cm dishes for growth to 60–70%

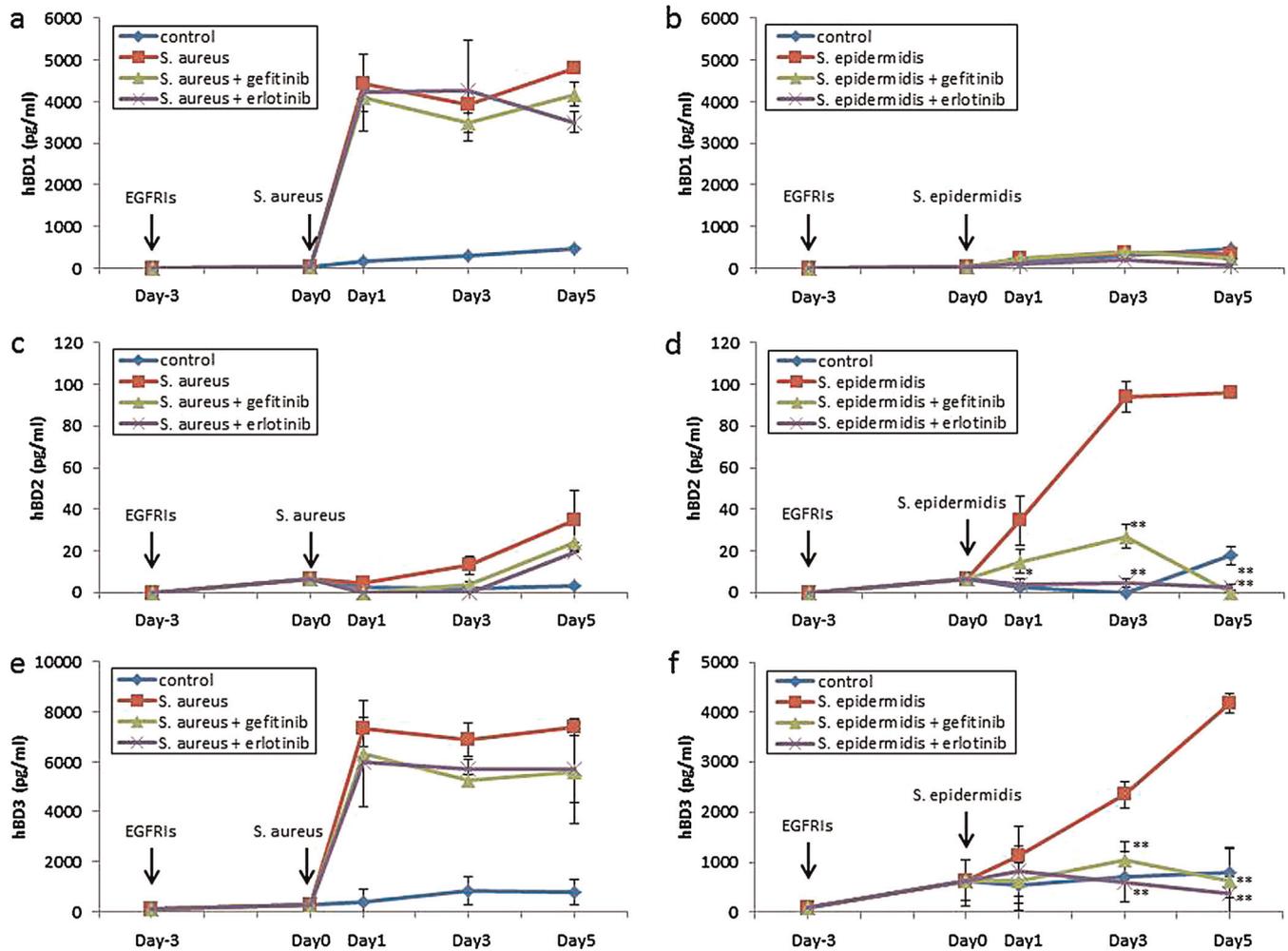


Fig. 2. Suppressive effects of EGFRIs on hBD expressions induced by *S. epidermidis* supernatants. NHEKs were cultured with or without EGFRIs for 3 days before stimulation with the bacterial supernatants, and the keratinocytes were cultured for additional 5 days. The expression levels of hBD1 (a and b), hBD2 (c and d) and hBD3 (e and f) in the culture supernatants were evaluated by ELISA. Data represent the means \pm standard deviations from three experiments. *P* values (bacteria stimulated keratinocytes cultured with EGFRIs vs. bacteria stimulated keratinocytes cultured without EGFRIs) were evaluated using Student's *t*-test (*S. aureus* or *S. epidermidis* vs. *S. aureus* + EGFRIs or *S. epidermidis* + EGFRIs, **p* < 0.05; ***p* < 0.01).

confluence. NHEKs were stimulated with staphylococcal supernatants at a final dilution ratio of 10%, or were stimulated with staphylococcal suspensions at a final density of 10^8 CFU/ml. We added the staphylococcal supernatants or suspensions by each medium change to keep the same concentrations. EGFRIs were added into the keratinocyte culture at various time points, i.e., 3 days before the stimulation, on the same day of the stimulation, and one day after the stimulation. All experiments were confirmed by using keratinocytes derived from several individuals.

2.4. Analysis of hBD expressions by enzyme-linked immunosorbent assay (ELISA)

Culture medium of NHEKs was sampled on day 0, 1, 3, and 5 of bacterial stimulation, and we analysed hBDs in the culture medium by ELISA. The hBDs were measured using hBD1 ELISA kit (Koma Biotech, Seoul, Korea), hBD2 ELISA kit (Phoenix Pharmaceuticals, Burlingame, CA, USA) and hBD3 ELISA kit (Adipo Bioscience, Santa Clara, CA, USA) according to the manufacturer's instructions. All samples were tested in duplicate. The ELISA results were expressed as pg/ml protein.

2.5. Statistics

Student's *t*-test was applied to determine calculating differences. *P*-value < 0.05 was defined statistically significance.

3. Results

3.1. The influence of bacterial supernatants on the expressions of hBDs from NHEKs

The supernatants of *S. aureus* induced the expressions of hBD1 (Fig. 1a) and hBD3 (Fig. 1c), but did not induce the expression of hBD2 (Fig. 1b). The supernatants of *S. epidermidis* induced the expressions of hBD2 and hBD3 but did not induce the expression of hBD1.

3.2. The effects of EGFRIs on hBD expressions induced by staphylococcal supernatants

We next examined the effects of EGFRIs on hBD productions from keratinocytes stimulated by staphylococcal supernatants. Both of two EGFRIs, gefitinib and erlotinib, suppressed the

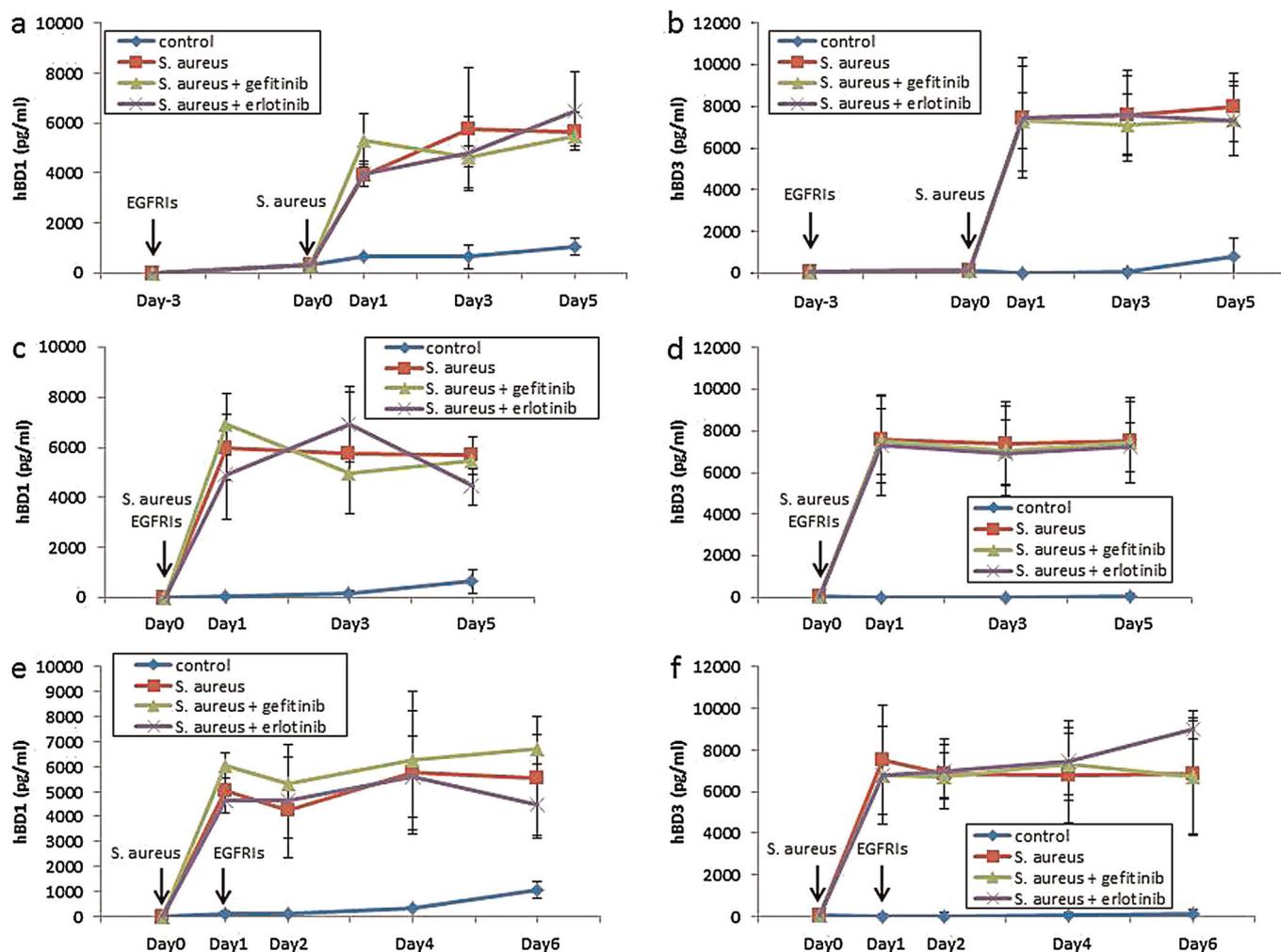


Fig. 3. Effects of EGFRIs applied at various timing on expression levels of hBD1 and hBD3 induced by *S. aureus*. EGFRIs were added into the keratinocyte culture at various time points, i.e., 3 days before the stimulation with *S. aureus* supernatants (a and b), on the same day of the stimulation (c and d), and 1 day after the stimulation (e and f). The expression levels of hBD1 (a, c, e) and hBD3 (b, d, f) in the culture supernatants were evaluated by ELISA. Data represent the means \pm standard deviations from three experiments. *P* values (bacteria stimulated keratinocytes cultured with EGFRIs vs. bacteria stimulated keratinocytes cultured without EGFRIs) were evaluated using Student's *t*-test (*S. aureus* vs. *S. aureus* + EGFRIs, **p* < 0.05; ***p* < 0.01).

expressions of hBD2 (Fig. 2d) and hBD3 (Fig. 2f) induced by *S. epidermidis*, and did not suppress the expressions of hBD1 (Fig. 2a) and hBD3 (Fig. 2e) induced by *S. aureus*.

3.3. The effects of EGFRIs on hBD1 and hBD3 induced by *S. aureus*

We assessed the effects of EGFRIs on the expressions of hBD1 and hBD3 induced by the supernatants of *S. aureus* with various timing of applying EGFRIs. EGFRIs did not suppress the expressions of hBD1 and hBD3 induced by *S. aureus* regardless of the timing of applying EGFRIs (Fig. 3a–f).

3.4. The effects of EGFRIs on hBD2 and hBD3 induced by *S. epidermidis*

We assessed the effects of EGFRIs on the expressions of hBD2 and hBD3 induced by the supernatants of *S. epidermidis* with various timing of applying EGFRIs. EGFRIs generally suppressed the expressions of hBD2 and hBD3 induced by *S. epidermidis* regardless of the timing of applying EGFRIs (Fig. 4a–d,f). Exceptionally, the suppression of hBD2 expression was not

observed by EGFRIs, which was added after the stimulation of *S. epidermidis* (Fig. 4e).

3.5. The effects of EGFRIs on hBD expressions induced by live *Staphylococci*

We examined the effects of EGFRIs on the expressions of hBD induced by the stimulation of live *S. aureus* and *S. epidermidis*. The results using live bacteria were basically similar to those using bacterial supernatants (Fig. 5a,b,d–f). The only difference between live bacteria and bacterial supernatants was that live *S. aureus* induced hBD2 expression and EGFRIs suppressed the expression, though the supernatants of *S. aureus* did not affect the expression of hBD2 (Fig. 5c).

4. Discussion

Human skin is selectively colonized by commensal bacteria, especially *S. epidermidis*; *S. aureus*, on the other hand, is rarely found on healthy human skin [6]. Expression of hBDs is known to be induced by inflammatory stimuli [7] and to actively

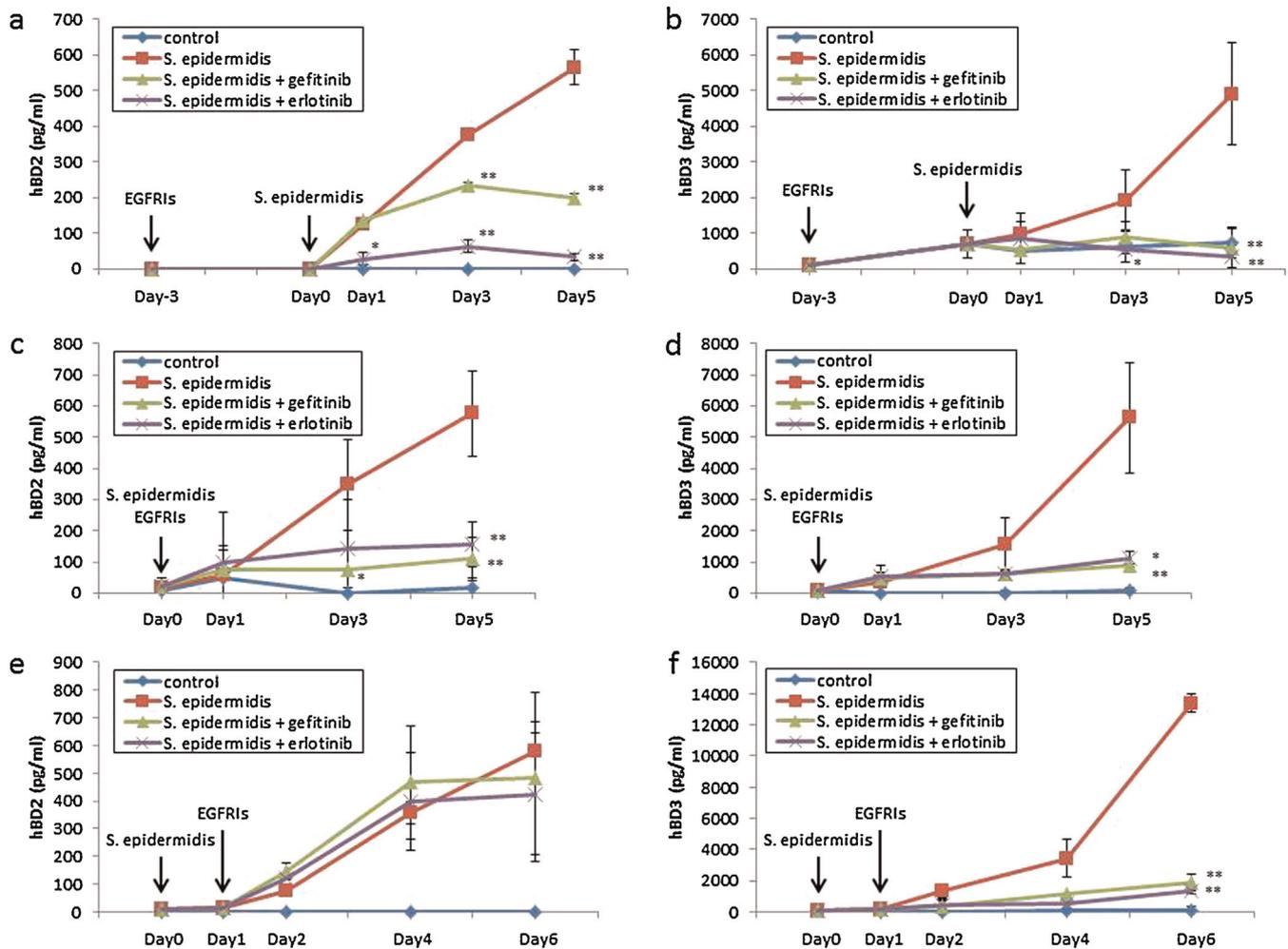


Fig. 4. Effects of EGFRIs applied at various timing on expression levels of hBD2 and hBD3 induced by *S. epidermidis*. EGFRIs were added into the keratinocyte culture at various time points, i.e., 3 days before the stimulation with *S. epidermidis* supernatants (a and b), on the same day of the stimulation (c and d), and 1 day after the stimulation (e and f). The expression levels of hBD2 (a, c, e) and hBD3 (b, d, f) in the culture supernatants were evaluated by ELISA. Data represent the means \pm standard deviations from three experiments. *P* values (bacteria stimulated keratinocytes cultured with EGFRIs vs. bacteria stimulated keratinocytes cultured without EGFRIs) were evaluated using Student's *t*-test (*S. epidermidis* vs. *S. epidermidis* + EGFRIs, **p* < 0.05; ***p* < 0.01).

contribute to host defense by inactivating microorganisms. Here, we observed EGFRIs suppressed the expressions of hBD2 and hBD3 induced by the supernatants or suspensions of *S. epidermidis*, while these drugs did not suppress the expressions of hBD1 and hBD3 induced by the supernatants or suspensions of *S. aureus*. These results demonstrated that EGFRIs suppress innate immune response that is maintained by *S. epidermidis*. *S. epidermidis* may limit the colonization and growth of pathogenic bacteria on human skin by inducing the expression of antimicrobial peptides [8]. EGFRIs decrease the levels of expression of hBD2 and hBD3 induced by *S. epidermidis*, which means that bacterial infection may occur, resulting in dermatologic toxicities such as follicular acneiform eruptions. EGFRIs may cause cutaneous adverse effects by perturbing the innate immune system that is maintained by commensal bacteria. We also observed that EGFRIs suppressed hBD2 expression induced by live *S. aureus*, which is also probably involved in cutaneous adverse effects by EGFRIs.

We previously demonstrated that the secreted products of *S. epidermidis* use the toll-like receptor (TLR) 2 pathway to induce hBD2 production from keratinocytes; the secreted products of

S. aureus, however, do not use these pathways in the induction of hBD1 and hBD3 production [9]. Furthermore, Wanke et al. indicated that the induction of hBD3 by secreted *S. aureus* products is independent of TLR2 and EGFR signalling, and the induction mediated by secreted factors of *S. epidermidis* involves TLR2 and EGFR signalling [10]. These previous findings are in accordance with the results of our present study, which led us to conclude that EGFRIs selectively suppress the expressions of hBDs induced by *S. epidermidis*. Since previous report suggested that EGFR-dependent mechanism regulate TLR2 [11], we consider EGFR may be an important regulator of immune response against bacterial infection by regulation of TLR2. We showed that live *S. aureus* increased the expression of hBD2 and EGFRIs suppressed the expression. Our previous study also revealed that not only live *S. epidermidis*, but also live *S. aureus*, could induce the production of hBD2 from keratinocytes, while the supernatants of *S. aureus* could not induce the production of hBD2 [9]. These results suggested that contact of keratinocytes with bacterial body of *S. aureus* may be necessary for the induction of hBD2 production from keratinocytes. Another report also showed that the expression of hBD2 mRNA by human keratinocytes was significantly induced by contact with heat-

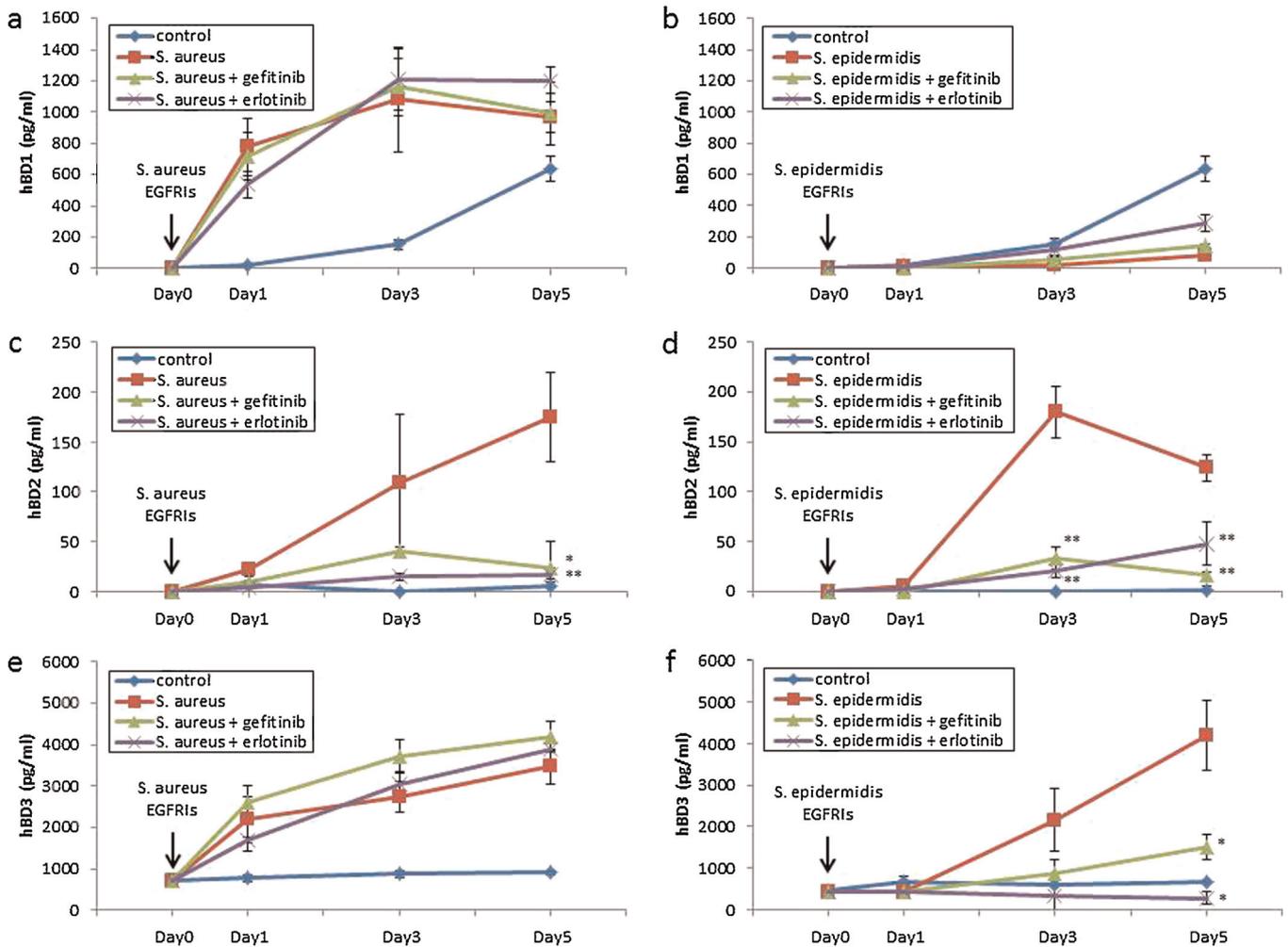


Fig. 5. Decreased live bacteria-induced expression levels of hBDs by EGFRi. EGFRi and live bacteria were added into NHEKs culture on the same time, and the keratinocytes were cultured for 5 days. The expression levels of hBD1 (a and b), hBD2 (c and d) and hBD3 (e and f) in the culture supernatants were evaluated by ELISA. Data represent the means \pm standard deviations from three experiments. *P* values (bacteria stimulated keratinocytes cultured with EGFRi vs. bacteria stimulated keratinocytes cultured without EGFRi) were evaluated using Student's *t*-test (*S. aureus* or *S. epidermidis* vs. *S. aureus* + EGFRi or *S. epidermidis* + EGFRi, **p* < 0.05; ***p* < 0.01).

inactivated *S. aureus* [12]. Further research is needed to verify whether the effects of EGFRi on the innate immune response induced by commensal bacteria are involved in cutaneous adverse reactions in the patients.

Conflict of interest

The authors have no conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jdermsci.2014.04.011>.

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