Selective induction of antimicrobial peptides from keratinocytes by staphylococcal bacteria

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ABSTRACT
Staphylococcus aureus (S. aureus) is one of the most clinically important inflammation-inducing pathogens, while Staphylococcus epidermidis (S. epidermidis) is nonpathogenic and hardly causes inflammation on skin. β-defensins, antimicrobial peptides, are secreted from keratinocytes constitutively or upon induction by various microorganisms. However, the difference between S. aureus and S. epidermidis is still unclear in terms of their influences on the production of β-defensins. In this study, we focused on the influences of S. aureus and S. epidermidis on the keratinocyte innate immune response. Pathogenic S. aureus mainly induced human β-defensin (hBD) 1 and hBD3, but not hBD2, and nonpathogenic S. epidermidis mainly induced hBD2 from human keratinocytes. Molecular weight fractions of >10 kDa prepared from S. aureus supernatants induced the production of hBD1 and hBD3. On the other hand, molecular weight fraction of >100 kDa prepared from S. epidermidis supernatants induced the production of hBD2. Furthermore, the secreted products of S. epidermidis used the toll-like receptor (TLR) 2 pathway in the induction of hBD2 production. The secreted products of S. aureus and S. epidermidis differentially induced subtypes of hBD through different receptors, which may be associated with the difference in virulence between these two bacteria.

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1. Introduction
Keratinocytes, which are continuously exposed to microorganisms from the external environment, operate as a front line of defense against the invasion of pathogenic microbes. It is expected that keratinocytes show various responses according to the kinds of microbes and act as a crucial site for innate immune response. Keratinocytes secrete not only a variety of cytokines, chemokines and growth factors, but also antimicrobial peptides, when stimulated by microorganisms [1]. Defensins are small cationic peptides that have antimicrobial activities and are secreted from epithelial cells, including keratinocytes. The β-defensins are cysteine-rich peptides of 36–42 amino acids in length and are stabilized by three disulfide bonds [2]. The three best-characterized human β-defensins (hBD), hBD1, hBD2 and hBD3, have been detected in human skin and cultured keratinocytes [3–5]. hBD1 expression is primarily constitutive, whereas the expression of hBD2 and hBD3 is inducible by cytokines, such as tumor necrosis factor (TNF)-α and IL-1β, various microorganisms, lipopolysaccharide and other microbial products [6–8]. The mechanism by which β-defensins kill or inactivate bacteria is not precisely understood but is generally thought to be a function of their pore-forming activity upon the microbial membrane [9]. It has been reported that β-defensin family members secreted from keratinocytes operate as immunoreactive agents in conjunction with antimicrobial peptides [10,11].

Staphylococcus aureus (S. aureus) is one of the most clinically important inflammation-inducing gram-positive pathogens. S. aureus is thought to be an exacerbating factor in atopic dermatitis (AD) and to have a negative influence on keratinocytes, while Staphylococcus epidermidis (S. epidermidis) is thought to be useful for maintenance of the cutaneous barrier function [12–14]. Components of gram-positive bacterial cell walls such as lipoteichoic acid (LTA) and peptidoglycan (PGN) can stimulate immunity. However, the specific involvement of keratinocytes in initiating and orchestrating immune responses to skin infections, including S. aureus colonization, is largely unknown.

It has been reported that the <10 kDa fraction of S. epidermidis induces hBD2 and hBD3 mRNA in undifferentiated human keratinocytes through toll-like receptor (TLR) 2 [15]. However, the difference between S. aureus and S. epidermidis is still unclear in terms of their influences on the production of hBD from...
keratinocytes. In this study, we focused on the influence of these two bacteria, *S. aureus* and *S. epidermidis*, on the keratinocyte innate immune response. We found that pathogenic *S. aureus* mainly induces hBD1 and hBD3, but not hBD2, and nonpathogenic *S. epidermidis* mainly induces hBD2, but not hBD1.

2. Materials and methods

2.1. The preparation of bacteria and their culture supernatants

*S. aureus*: IID980, JCM2151, 2179, 2413 and clinical isolates (two strains of MSSA), and *S. epidermidis*: ATCC12228, JCM2414 and two strains of clinical isolates (*S. epidermidis-1* and *S. epidermidis-2*), were used for the experiment. Staphylococcal clinical isolates were obtained from the central clinical laboratory of Nara Medical University Hospital. Clinical isolates of *S. aureus* and *S. epidermidis* were confirmed by 16S rRNA sequences as previously described [16]. Other staphylococcal strains (*S. aureus*: JCM2151, 2179 and 2413, 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 10^9 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 μm Millipore filter unit (Millipore, Billerica, MA, USA) and stored at −30 °C until use. Amicon Ultra Centrifugal Filter Units 10 kDa and 100 kDa (Millipore, Billerica, MA, USA) were used to fractionate the bacterial supernatants. We firstly separated the bacterial supernatants with 100 kDa filter unit to prepare molecular weight fractions of >100 kDa and <100 kDa. Next, the supernatant fraction of <100 kDa was again separated with the 10 kDa filter unit to prepare the fractions of <10 kDa and 10–100 kDa, and stored at −30 °C until use.

2.2. 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 µg/ml), hydrocortisone (0.5 µg/ml), gentamycin (50 µg/ml), amphotericin B (50 ng/ml) and bovine brain pituitary extract (0.4% v/v) at 37 °C in 5% CO2. NHEKs at passage three or four were seeded to 24-well culture plates for growth to 60–70% confluence. NHEKs were stimulated with live staphylococci at a density of 10^8 CFU/ml for 24 h. The cells were also stimulated with culture supernatants of staphylococci at a final dilution ratio of 1/10 for 24 h. All experiments were confirmed by using keratinocytes derived from several individuals.

2.3. Inhibition of TLR2 by RNA interference

NHEKs were seeded to 24-well culture plates (5 x 10^4 cells/well) and incubated in antibiotic-free medium overnight at 37 °C in 5% CO2 prior to transfection. The cells were transfected with 10 nM small interfering RNA (siRNA) targeted to TLR2 and non-targeted control siRNA (Silencer® GUCta-3'; 5'CUAUAAUUACUtt-3'; Silencer® Select Negative Control siRNA by using Lipofectamine™ RNAiMAX Reagent (Life Technologies Corporation, Carlsbad, CA, USA)) according to the manufacturer's protocol. TLR2 siRNA (sense: 5'-GACUUAUCUAAUAUACUt-3'; antisense: 5'-AGUAUUAUGAGAAUGUa-GUCta-3'; Silencer® Select Pre-designed TLR2 siRNA) and negative control siRNA (Silencer® Select Negative Control No.1 siRNA) were purchased from Life Technologies Corporation. Following 24 h of transfection, NHEKs were stimulated with culture supernatants of S. aureus and S. epidermidis for 24 h.

2.4. RNA extraction and real-time PCR

Total RNA from transfected cells was isolated using PureLink™ RNA Micro kit (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions, and concentrations were measured by spectrophotometry (GeneQuant pro, Biochrom Ltd., Cambridge, UK). RNA quality was assessed by measuring the ratio of absorbance at 260/280 nm. For generation of first-strand cDNA, 1 µg of total RNA was used in a reverse transcription reaction using SuperScript VILO™ MasterMix (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. Next, real-time PCR was carried out in a final volume of 20 µl containing cDNA template, TLR2 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Life Technologies Corporation, Carlsbad, CA, USA), TaqMan® Fast Advanced MasterMix (Life Technologies Corporation, Carlsbad, CA, USA), and DNase RNAase free water, using a StepOne™ real-time PCR system (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol. The primers and TaqMan® probe sets for human TLR2 (Hs00006101_m1) and human GAPDH endogenous control (Hs99999905_m1) were purchased from Life Technologies Corporation. Amplification conditions were as follows: 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. TLR2 mRNA expression was measured by real-time PCR using the ΔΔCt method.

2.5. Enzyme-linked immunosorbent assay (ELISA)

We measured hBD in the culture supernatants of NHEKs after stimulation with live or supernatants of staphylococci by ELISA. hBD 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

![Fig. 2](image-url)

The production of hBDs from human keratinocytes stimulated with different molecular weight fractions of the supernatants of staphylococci. NHEKs were cultured with different molecular weight fractions derived from the supernatants of staphylococci for 24 h: (a) hBD1, (b) hBD2 and (c) hBD3 proteins in the culture supernatants of NHEKs were measured by ELISA. The data are means ± standard deviations from three independent experiments.
tested in duplicate. The ELISA results were expressed as pg/ml protein.

3. Results

3.1. The influence of live S. aureus and S. epidermidis on the production of hBD from NHEKs

All of the strains of S. aureus induced the production of hBD1 from NHEKs; however, no strains of S. epidermidis could induce the production of hBD1 (Fig. 1a). Similarly to the production of hBD1, stimulation of NHEKs with S. aureus induced the production of hBD3, and stimulation with S. epidermidis induced very low or no production of hBD3 (Fig. 1c). On the other hand, the production of hBD2 was increased by stimulation with both S. aureus and S. epidermidis (Fig. 1b).

3.2. The influence of bacterial supernatants on the production of hBD from NHEKs

The supernatants of S. aureus induced the production of hBD1 from NHEKs; however, the supernatants of S. epidermidis could not induce the production of hBD1 from NHEKs (Fig. 1d). Similarly to the production of hBD1, stimulation with the supernatants of S. aureus also induced the production of hBD3 (Fig. 1k). On the other hand, the production of hBD2 was increased by the stimulation with the supernatants of S. epidermidis, but not by the stimulation with the supernatants of S. aureus (Fig. 1e).

Next, different molecular weight fractions derived from the supernatants of staphylococci were exposed to NHEKs to examine which molecular weight fraction influences the production of hBD from NHEKs. Molecular weight fractions of 10–100 kDa and >100 kDa prepared from S. aureus supernatants, but not the fraction of <10 kDa, induced the production of hBD1 and hBD3 from NHEKs (Fig. 2a and c). On the other hand, molecular weight fraction of >100 kDa prepared from S. epidermidis supernatants mainly induced the production of hBD2 from NHEKs (Fig. 2b).

3.3. The induction of hBD by S. epidermidis through TLR2 and by S. aureus through a pathway other than TLR2

We next examined the mechanism involved in the recognition of staphylococci that induces the production of hBD. To block TLR2-dependent activation, siRNA targeted to TLR2 was transfected to NHEKs, and then the supernatants of S. aureus and S. epidermidis were added to the culture system to stimulate the NHEKs. The knockdown of TLR2 mRNA in NHEKs transfected with TLR2 siRNA was confirmed by real-time PCR, and a 75% knockdown of TLR2 mRNA was observed. Targeted knockdown of TLR2 by siRNA abrogated the induction of hBD2 secretion by S. epidermidis products (Fig. 3b). In contrast, TLR2 knockdown by siRNA did not
abrogate hBD1 and hBD3 secretions by S. aureus products (Fig. 3a and c). Furthermore, only secreted products of S. epidermidis, not secreted products of S. aureus, increased the TLR2 mRNA expression in NHEKs (Fig. 3d). These data demonstrated that secreted products of S. epidermidis, but not secreted products of S. aureus, induce hBD through TLR2 signaling.

4. Discussion

In this study, we observed that the secreted products of S. aureus and S. epidermidis differentially induced the subtypes of hBD from keratinocytes. That is, the supernatants of S. aureus induced the production of hBD1 and hBD3, but not hBD2, while the supernatants of S. epidermidis strongly induced the production of hBD2. On the other hand, 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. These results suggested that contact of keratinocytes with the bacterial body of S. aureus, but not contact with secreted products of S. aureus, may be necessary for the induction of hBD2 production from keratinocytes. A previous report also showed that the expression of hBD2 by keratinocytes was induced consistently by live S. aureus and S. epidermidis [17], and another report described that the expression of hBD2 mRNA by human keratinocytes was significantly induced by contact with heat-inactivated S. aureus [1]. These two reports support our present finding.

Lai et al. [15] reported that the exposure of cultured keratinocytes to the supernatants of S. epidermidis enhanced hBD3 mRNA expression more than the supernatants of S. aureus did. On the other hand, our results showed that the supernatants of S. aureus induced hBD3 production from keratinocytes much more than the supernatants of S. epidermidis did. Although the reason for this discrepancy is not completely clear at present, secreted hBD3 protein detected by ELISA may not reflect mRNA expression for some reason, such as low efficiency of hBD3 secretion in keratinocytes stimulated by S. epidermidis.

S. aureus is pathogenic and causes inflammation on the skin, while S. epidermidis is nonpathogenic and hardly causes inflammation on the skin. It is well known that the β-defensins contribute to host defense by killing invading pathogenic microorganisms. In addition, recent study has shown that the β-defensins play an important role in chemotaxis and activation of inflammatory cells [14,18]. Therefore, S. aureus-induced hBD1 and hBD3 might be involved in the development of inflammation in cutaneous S. aureus infection.

In this study, we observed that a high-molecular-weight fraction over 100 kDa prepared from S. epidermidis supernatants had activity to induce hBD2, but a lower-molecular-weight fraction did not. However, a previous report demonstrated that the exposure of cultured keratinocytes to a small molecule (<10 kDa) from S. epidermidis supernatants enhanced hBD2 mRNA expression [15]. The reason for this discrepancy is unknown at present. We also observed that the molecular weight fractions of 10–100 kDa and >100 kDa prepared from S. aureus supernatants had activity to induce hBD1 and hBD3. These results and induction pattern of hBD subtypes suggest that the products inducing hBD are different between S. epidermidis and S. aureus.

Furthermore, we observed that the secreted products of S. epidermidis used the TLR2 pathway for the induction of hBD2 production from keratinocytes, as Lai et al. previously described, while the secreted products of S. aureus did not use these pathways in the induction of hBD1 and hBD3 production.

Taken together, we found that the secreted products of S. aureus and S. epidermidis differentially induced subtypes of hBD form human keratinocytes through different receptors. Further investigation should be undertaken to elucidate the pathogenetic implication of these different innate immune responses to two types of Staphylococcal bacteria.

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References


