Roles of SEA-expressing *Staphylococcus aureus*, isolated from an atopic dermatitis patient, on expressions of human ß-defensin-2 and inflammatory cytokines in HaCaT cells

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Abstract. Atopic dermatitis (AD) shows an increased susceptibility to *Staphylococcus aureus* infection partly due to decreased expression of human ß-defensin-2 (HBD-2). Interestingly, it was reported that the nasal carrier *S. aureus* down-regulates the expression of HBD-2 and -3, thereby the carrier strains of *S. aureus* retain an advantage to epithelial colonization and infection. In this study, we tried to isolate and characterize *S. aureus* from an AD patient, with recurrent oozing on his face. We studied the increased expression of inflammatory cytokines, such as IL-1ß, -6, -8, and TNF-α in *S. aureus* treated-HaCaT cells, which are mediated by secreting superantigens (SAgs), structural component, or both. In addition, we investigated whether the SAgs from *S. aureus* can down-regulate the expression of HBD-2 in HaCaT cells making favorable conditions for colonization on skin. Our data showed that the isolated *S. aureus* has the exotoxin gene, *sea* exotoxin. The SEA producing-*S. aureus* induced the expression of IL-1ß, -6, -8 cytokines, and TNF-α in HaCaT cells. The expression of HBD-2 was increased in *S. aureus*-treated HaCaT cells. Furthermore IL-8 was also induced by the structure component of *S. aureus*. Taken together, the SEA producing *S. aureus* induced the up-regulation of pro-inflammatory cytokines as well as HBD-2, thereby resulting in induction of the persistent eczematous skin lesions in AD. Thus, our data may give insight into understanding the pathogenesis by which *S. aureus* induces and aggravates eczematous skin lesions in AD.

Introduction

Atopic dermatitis (AD) is an inflammatory skin disease with a course of remission and exacerbation (1). When AD is exacerbated by several factors including infection and dryness, acute erythematous eczema lesions are sometimes observed in the skin, characterized by erythema, oozing and crusting (2,3).

Staphylococcus aureus expressing superantigens (SAgs) induce eczematoid skin reactions in AD patients (4,5). Over half of the *S. aureus* strains isolated from AD skin exhibited the secretion of superantigen toxins, including staphylococcal enterotoxins A and B (SEA, SEB), and toxic shock syndrome toxin-1 (TSST-1), which induces the pro-inflammatory effects through secretion of inflammatory cytokines such as IL-1ß, -6, -8, and TNF-α. These cytokines induce persistent eczema in AD (6-8). However, it is still unknown if the increased expression of pro-inflammatory cytokines are mediated by both the secreting SAgs and structural component of *S. aureus*.

The antimicrobial peptides play roles in inhibiting the colonization of *S. aureus*. The colonization of *S. aureus* is inhibited by human ß-defensin-2 (HBD-2) antimicrobial peptide (9). It was reported that the nasal carrier *S. aureus* down-regulates the expression of HBD-2 and -3, thereby the carrier strains of *S. aureus* retain an advantage to epithelial colonization and infection (10). It is important to investigate whether *S. aureus* induces the decreased expression of HBD-2, thereby resulting in more susceptible local conditions for colonization of *S. aureus* and persistence of eczema in AD.

Thus, in this study, we tried to isolate and characterize *S. aureus* from an AD patient, with recurrent oozing on the face. We studied the increased expression of inflammatory cytokines, such as IL-1ß, -6, -8, and TNF-α in *S. aureus* treated-HaCaT cells, mediated by secreting SAgs, structural component, or both. In addition, we investigated whether the SAgs from *S. aureus* can down-regulate the expression of HBD-2 in HaCaT cells to make favorable conditions for colonization on the skin.

Materials and methods

Patient information. The patient, a 17 year-old man, has suffered from AD since childhood. He has dry skin on the trunk with a lichenified patch on the elbow, around the neck, and face. He frequently suffers from recurrent yellowish discharge and oozing of eczematoid on his face. We treated him with saline wet dressing on his face and topical antibiotic creams. This was followed by systemic antibiotics, antihistamines, systemic steroids, and topical steroid creams.

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Isolation and characterization of S. aureus. The bacteria were isolated from eczematous skin lesions of AD by sterile swabs. The bacteria samples were plated into a nonselective blood agar plate and in addition onto the mannitol agar plate containing 7.5% sodium chloride to improve detection of *S. aureus*. The SAgs toxin genes, such as enterotoxin genes (*sea*, *seb*, *sec*) and exfoliative toxin genes (*eta*, *etb*) were detected by multiplex PCR as described previously (11). Bacteria supernatants were prepared by growing bacteria in tryptic soy broth (TSB) medium for 16-18 h at 37˚C under agitation (120 rpm). Supernatant of $5x10^8$ cfu/ml at stationary phase was obtained by centrifugation at 960 g for 10 min at 4˚C, followed by filtration through a 0.2 μm filter. The supernatant corresponding to *S. aureus* soluble SAgs was diluted at 20% in culture media. TSB medium was used as control at a 20% dilution in culture media.

Cell culture. Human keratinocyte cell line, HaCaT cells, were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 in Eagle's minimum essential medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin. For experiments, cells ($5x10^4$ cells/ml) were seeded in a culture dish and maintained in a tissue culture incubator.

Examination of cell morphology. Cells were fixed with 95% cold-ethanol for 4 min and washed twice with PBS. After fixation, cells were stained with staining solution [PI (1 μg/ml), RNase A (0.1 mg/ml)] for 20 min at room temperature. Nuclear morphology of PI-stained cells was observed under fluorescence microscope (Olympus Optical, Tokyo, Japan). The cells were also stained with crystal violet solution for 10 min and washed three times by phosphate buffered saline (PBS) and the morphology of cells was observed under a phase contrast microscope.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from cells using the RNAzol™ B (Biotecx Laboratories, Houston, TX) according to the manufacturer's instructions and quantitated by spectrophotometer. Total RNA (1 μg) was reverse transcribed using M-MLV reverse transcriptase (Promega Co., Madison, WI). The PCR reaction was carried out following the manufacturer's instructions (Takara Co., Otsu, Japan). The primer sequences and product sizes were as follows: 1) GAPDH (forward, 5'-CGT CTT CAC CAC CAT GGA GA-3', reverse, 5'-CGG CCA TCA CGC CAC AGT TT-3'), 300 base pair (bp); 2) IL-1ß (forward, 5'- AAA AGC TTG GTG ATG TCT GG-3', reverse, 5'-TTT CAA CAC GCA GGA CAG G-3'), 179 bp; 3) IL-6 (forward, 5'-GTG TGA AAG CAG CAA AGA GGC-3, reverse, 5'-CTG GAG GTA CTC TAG GTA TAC-3'), 159 bp; 4) IL-8 (forward, 5'- ATG ACT TCC AAG CTG GGC CGT G-3', reverse, 5'-TAT GAA TTC TCA GCC CTC TTC AAAA-3'), 301 bp. 5) TNF-α (forward, 5'- CAA AGT AGA CCT GCC CAG AC-3', reverse, 5'-GAC CTC TCT CTA ATC AGC CC-3'), 490 bp. 6) HBD-2 (forward, 5'-ATC TCC TCT TCT CGT TCC TC-3', reverse, 5'-ACC TTC TAG GGC AAA AGA CT-3'), 126 bp.

Western blot analysis. Whole cell extracts were prepared in the lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, phenylmethylsulphonyl fluoride (PMSF), aprotinin, leupeptin (10 mg/ml of each), 5 mM phenanthroline and 28 mM benzamidine-HCl]. For phospho-protein detection, cells were washed with ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4 and 1 mM NaF, and lysed in a buffer [20 mM Tris-Cl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF, 2 mM EDTA, 200 nM aprotinin, 20 μM leupeptin, 50 μM phenanthroline, 280 μM benzamidine-HCl]. Equal amounts of protein (40 μg/lane) were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was then washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl) containing 0.05% Tween-20 (TBST) and blocked in TBST containing 5% non-fat dried milk. The membrane was further incubated with respective specific antibodies such as p-ERK (1:2000), ERK (1:2000), p-JNK (1:1000), JNK (1:2000), p-p38 (1:1000), p38 (1:2000), and ß-tubulin (1:5000). The membrane was continuously incubated with appropriate secondary antibodies coupled with horseradish peroxidase and developed in the ECL Western detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

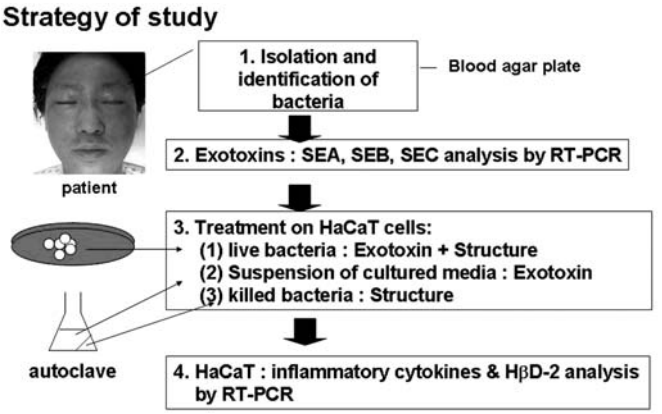

Figure 1. The schematic presentation of this study. The bacteria was isolated from eczematoid skin and characterized by culture media, and the exotoxin genes were specifically amplified by multiplex PCR analysis. The effect of bacteria on expression of inflammatory cytokines and HBD-2 were studied by RT-PCR analysis in live, filtered suspension, or killed bacteria-treated HaCaT cells.

Results

Isolation of SEA expressing S. aureus. The AD patient clearly showed erythema and eczema with oozing on the face. To isolate and characterize the bacteria on skin lesions, we planned a schematic strategy (Fig. 1) and two swabs were taken according to standard procedures (12). One swab was from an eczematous skin lesion and the other from the nose. The *S. aureus* was isolated from eczematous skin lesion (Fig. 2A) and the isolated *S. aureus* was of a methicillin-sensitive strain (MSSA) (Fig. 2B). The other isolated *S. aureus* strain from the nose was also MSSA (data not shown). The toxin gene (*sea*) was detected in isolated *S. aureus* by multiplex PCR but other toxin genes such as *seb*, *sec*, and exfoliative toxin genes (*eta*, *etb*) were not detected (Fig. 3). To determine the cytotoxic effect of the SEA expressing *S.*

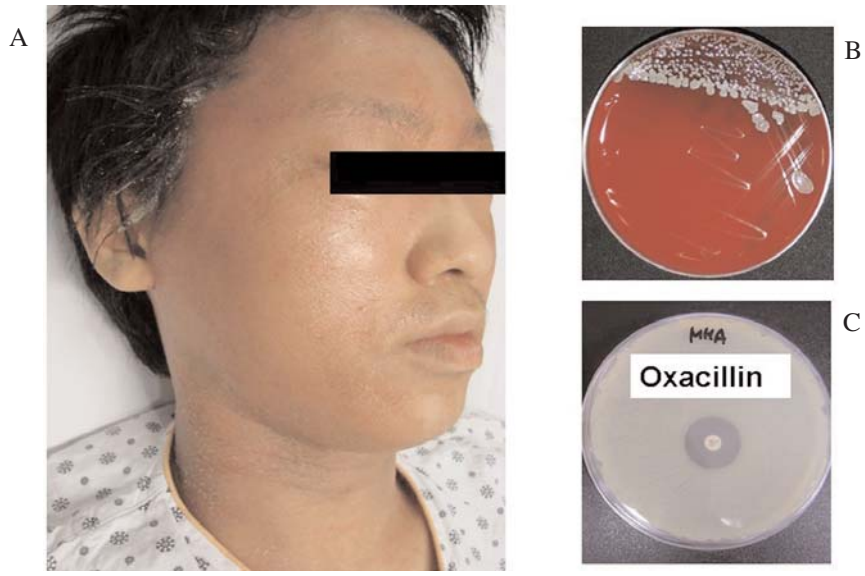

Figure 2. Isolation and culture of *S. aureus* from an atopic dermatitis patient. (A) Eczematous erythema and yellowish crusts with oozing on the face observed in atopic dermatitis patient. (B) The bacterial colonies were cultured on a blood agar plate and (C) bacteria showed susceptibility to oxacillin.

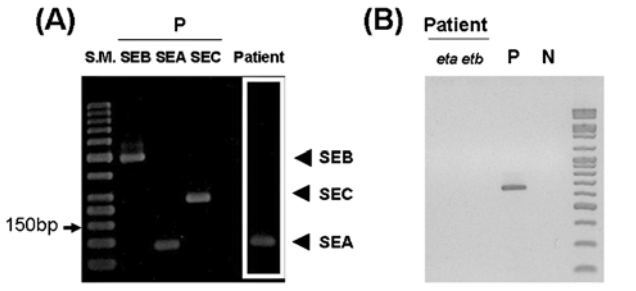

Figure 3. Detection of superantigens and exfoliative toxin genes in *S. aureus*. (A) The SEA gene, but not SEB and SEC, was clearly amplified by multiplex PCR and (B) both exfoliative toxin genes (ETA and ETB) were not detected by PCR analysis. P, positive control; N, negative control.

aureus, we cocultured HaCaT cells with *S. aureus*. The morphology of HaCaT cells shrank slightly, similar to cytotoxic damaged cells and the fragmented nucleus was observed under a fluorescence microscope (Fig. 4).

Up-regulation of inflammatory cytokines by S. aureus-treated HaCaT cells. To analyze the effect of the structural component of *S. aureus* and SAgs on expression of inflammatory cytokines, we treated live *S. aureus* itself, filtered (0.02 μm acrodisc) supernatant, or killed-*S. aureus* by autoclave.

First, HaCaT cells were exposed to *S. aureus* and harvested 24 h after treatment for RT-PCR analysis. As shown in Fig. 5, IL-1ß, -6, -8, and TNF-α expressions were dramatically increased by *S. aureus* treatment. To clarify the roles of the

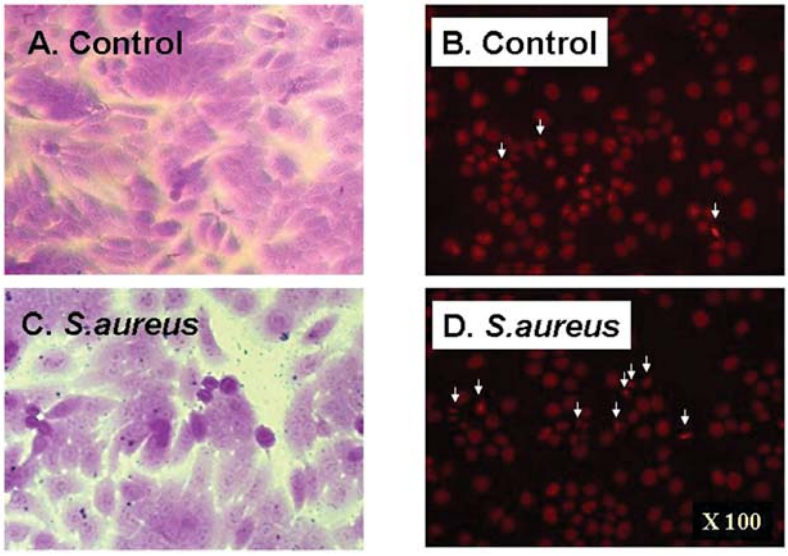

Figure 4. Apoptotic cellular and nuclear morphologies of HaCaT cells. HaCaT cells were cocultured with *S. aureus* in DMEM for 24 h. The morphologies of HaCaT cells cocultured with *S. aureus* were shrunken and changed to a round shape (arrow), as compared to control (C). The nucleus was markedly fragmented in HaCaT cells cocultured with *S. aureus* (arrow), as compared to control (D).

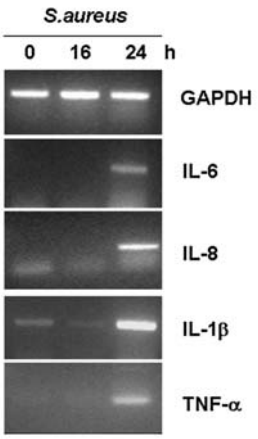

Figure 5. Increased expressions of inflammatory cytokines in HaCaT cells cocultured with *S. aureus*. The IL-1ß, -6, -8, and TNF-α mRNA were specifically amplified by PCR primers.

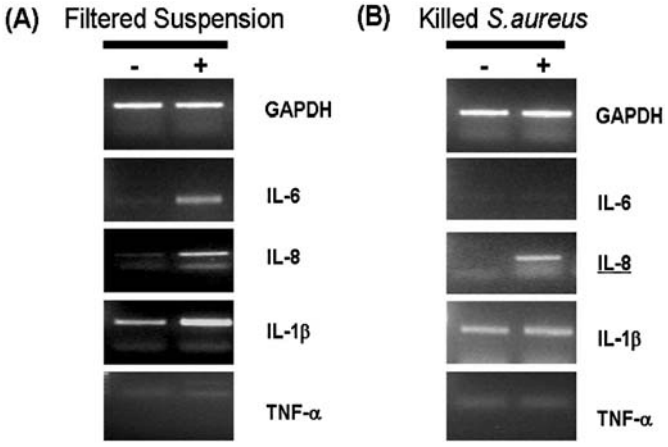

Figure 6. Comparison of expression of inflammatory cytokines between in suspension-treated and autoclaved *S. aureus*-treated HaCaT cells. (A) HaCaT cells were treated with culture media of *S. aureus* or (B) autoclaved-*S. aureus*, and then the IL-1ß, -6, -8, and TNF-α mRNA were specifically amplified by PCR primers.

secreting SAgs or the structural components of *S. aureus* on the expression of pro-inflammatory cytokines, HaCaT cells were treated by suspension of bacterial cultures, filtered by 0.02 μm acrodisc, or killed bacteria by autoclave. The expression of IL-1ß, -6, and -8, but not TNF-α, were increased by the treatment of filtered suspension of bacterial cultures, implying the SAgs induce the expression of IL-1ß, -6, and -8 in HaCaT cells (Fig. 6A). Interestingly, the killed bacteria did not induce the expression of pro-inflammatory cytokines except for IL-8 (Fig. 6B). These results indicate that the increased expression of IL-1ß, -6, -8, and TNF-α in *S. aureus*-exposed HaCaT cells are mainly regulated by the secretion products of *S. aureus*, which may be SAgs (SEA). Further studies are required to elucidate the component of suspension of bacteria, inducing inflammatory cytokine expression.

Up-regulation of HBD-2 by S. aureus-treated HaCaT cells. It is unknown whether the *S. aureus* down-regulates the expression of HBD-2 in keratinocytes to facilitate the skin infection,

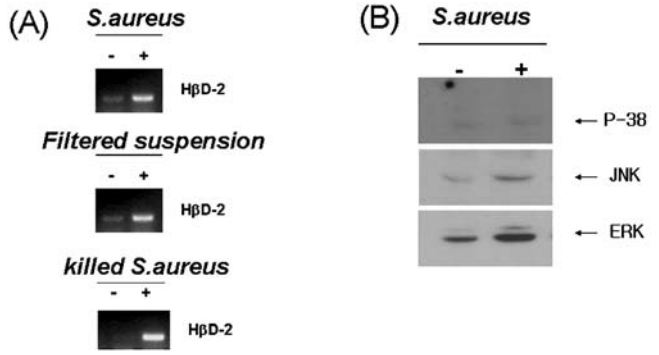

Figure 7. (A) Increased expressions of HBD-2 and (B) activation of JNK and ERK in HaCaT cells by *S. aureus*.

especially in AD patients showing recurrent erythema and oozing on the face. As shown in Fig. 7A, *S. aureus* did not down-regulate the expression of HBD-2 in HaCaT cells. We clearly observed that expression of HBD-2 was markedly up-regulated in HaCaT cells by *S. aureus*, implying down-regulation of HBD-2 in AD is regulated by other key factors, but not by *S. aureus* itself.

The expression of HBD-2 is regulated by p38 MAPK or JNK in cells. As shown in Fig. 7B, the activation of JNK and ERK were observed in the *S. aureus* treated HaCaT cells, and stripping and reprobing the same membrane with antibodies against JNK and ERK revealed no change in total protein levels of each kinase (data not shown), indicating that the expression of HBD-2 is regulated partly by the *S. aureus*-induced activation of pre-existing JNK and ERK.

Discussion

The increased colonization of *S. aureus* in the lesional skin in AD patients contributes to the severity of eczema (4,5). Leung *et al* reported that *S. aureus* isolated from lesional skin of more than half of their patients with AD could secrete toxins with the characteristics of SAgs (13). The SAgs of *S. aureus* are composed of enterotoxins (SEA, SEB, SEC), exofoliative toxins (ETA, ETB), and TSST-1 (14). Furthermore, these SAgs trigger the immunologic responses mediated by cytokines, resulting in inflammation of tissues (6-8,15). Thus, it is accepted that several SAgs are associated with more extensive lesions and the presence of eczema in AD.

In this study, we reported the isolation and characterization of *S. aureus*, which is colonized in the AD patient showing recurrent eczema on the face for 3 years. The isolated *S. aureus* has the toxin gene, *sea*, but not *seb*, *sec*, *eta*, and *etb*. Our data clearly show that the expression of inflammatory cytokines including IL-1ß, -6, -8, and TNF-α, were increased by *S. aureus*. In addition, it was reported that in organ-cultured human skin, the SAgs could induce cytokines (TNF-α, IL-1α, and -1ß) and among these cytokines, TNF-α was the most abundantly expressed in the keratinocytes at both the protein and mRNA levels (16). Both IL-1ß and TNF-α play roles as potent primary stimuli for IL-6 and -8 production (17). The local cytokine levels of TNF-α, IL-1ß, -6 and -8 were markedly high in severe exanthematous diseases in newborn infants caused by exotoxins producing methicillin-resistant *S. aureus*

(18). Interestingly, in this study, our data show that the expression of IL-8, but not IL-1ß, TNF-α, and IL-6, was up-regulated by the treatment of autoclaved-*S. aureus*. These data indicate that the expression of IL-8 is regulated by the structure of *S. aureus* as well as secretion products such as exotoxins. It was reported that lipoteichoic acid from *S. aureus* induced a strong release of the chemoattractants, LTB4, IL-8, C5a, MCP-1, and the colony-stimulating factor G-CSF in whole blood, playing roles in stimulus for neutrophil recruitment (19). Taken together, the SAgs and structure of *S. aureus* such as lipoteichoic acid have been shown to exert direct pro-inflammatory effects on keratinocytes via TNF-α or IL-1ß secretion, resulting in induction and persistent eczematous skin lesions in AD.

The AD patients are highly susceptible to cutaneous bacterial, fungal, and viral infections (1,2). The decreased expression of antimicrobial peptides, such as HBD-2 and -3, partly play roles in the underlying molecular mechanisms of the increased susceptibility of bacterial cutaneous infections in AD (9). However, the precise molecular mechanisms of the decreased expression of HBD-2 in AD are still unknown. Recently, it was reported that the nasal carrier *S. aureus* delayed the expression of the pattern recognition receptor, Toll-like receptor 2 (TLR2) and furthermore down-regulates the expression of HBD-2 and -3, thereby the carrier strains of *S. aureus* retain an advantage by delaying the host's innate response to epithelial colonization and infection (10). Our patient also had nasal *S. aureus*, producing the SAgs, and showed recurrent eczematous skin lesions on the face. Thus, we hypothesized that the colonization of the SAg-producing *S. aureus* down-regulates the expression of HBD-2 in AD for facilitating the cutaneous skin. In this study, our data clearly show that the expression of HBD-2 was markedly increased by the live and cultured media of *S. aureus*, or killed *S. aureus*-treated HaCaT cells. Thus, SAg-producing *S. aureus* did not down-regulate the expression of HBD-2 in HaCaT cells. Recently, it was suggested that the increased expression of IL-4, -13, and -10 in AD lesions may contribute to the decreased expression of HBD-2 by down-regulation of cytokine expression, which has the capability to induce the HBD-2 expression. To clarify the roles of SAg-producing *S. aureus* in eczema, further studies should be performed among the expression levels of pro-inflammatory cytokines, HBD-2, and colonization density of SAg-producing *S. aureus* in eczematoid skin in AD.

In conclusion, we demonstrated the SAg-producing *S. aureus* from an AD patient with recurrent oozing on the face. The same strain was also colonized in the nostril of the AD patient. SAg-producing *S. aureus* induce pro-inflammatory cytokines, such as IL-1ß, -6, and -8 as well as HBD-2 in HaCaT keratinocytes through secretion of SEA, thereby resulting in induction and persistent eczematous skin lesion in AD. Furthermore, the expression of IL-8 is also induced by the structure component of *S. aureus*. Thus, our data may give insight into understanding the pathogenesis by which *S. aureus* induces and aggravates eczematous skin lesions in AD.

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