Lipoteichoic Acid Isolated from *Staphylococcus aureus* Induces Both Epithelial-Mesenchymal Transition and Wound Healing in HaCaT Cells

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Lipoteichoic acid (LTA), a cell wall component of gram-positive bacteria, is recognized by Toll-like receptor 2, expressed on certain mammalian cell surfaces, initiating signaling cascades that include nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase. There are many structural and functional varieties of LTA, which vary according to the different species of gram-positive bacteria that produce them. In this study, we examined whether LTA isolated from *Staphylococcus aureus* (aLTA) affects the expression of junction proteins in keratinocytes. In HaCaT cells, tight junction-related gene expression was not affected by aLTA, whereas adherens junction-related gene expression was modified. High doses of aLTA induced the phosphorylation of extracellular signal-regulated protein kinases 1 and 2, which in turn induced the epithelial-mesenchymal transition (EMT) of HaCaT cells. When cells were given a low dose of aLTA, however, NF-κB was activated and the total cell population increased. Taken together, our study suggests that LTA from *S. aureus* infections in the skin may contribute both to the outbreak of EMT-mediated carcinogenesis and to the genesis of wound healing in a dose-dependent manner.

Keywords: Lipoteichoic acid, *Staphylococcus aureus*, epithelial-mesenchymal transition, keratinocytes, Toll-like receptor, wound healing

Introduction

Staphylococcus aureus is a gram-positive bacterium frequently found on skin. When pathogenic, it can cause various skin diseases such as cellulitis, folliculitis, impetigo scald skin syndrome, and atopic dermatitis. Skin diseases can occur as a result of exogenous factors, as well as environmental changes or changes in the ratio of *S. aureus* to other microflora on the skin [1, 2]. In general, lipoteichoic acid (LTA) from gram-positive bacteria activates a wide variety of signaling cascades, including those involving nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK), through interaction with Toll-like receptor (TLR) 2, which results in the induction of pro-inflammatory cytokine

secretion. However, different strains and species of gram-positive bacteria have their own distinct LTA isomers with varying structures, which leads to diverse physiological effects against mammalian immune cells [3, 4].

Cell junctions, such as tight junctions, adherens junctions, and gap junctions, bind epithelial cells to each other, building up the paracellular barrier and controlling paracellular transport and communication between neighboring cells [5]. Claudins are a typical family of proteins that comprise tight junctions, whereas E-cadherin and connexins are the main constituents of adherens junctions and gap junctions, respectively. The variation in the expression pattern of E-cadherin acts as an epithelium-mesenchymal transition (EMT) marker. Cells with an epithelial phenotype normally secrete high levels of E-cadherin, but this expression is

decreased during EMT. On the other hand, cells with a mesenchymal phenotype express N-cadherin and Snail [6]. In vertebrates, tight junctions are also known as occluding junctions. They join the membranes of neighboring cells to form a virtually impermeable barrier, which serves to maintain the osmotic balance of the cells. Adherens junctions are an intermediate junction, which serve as a bridge connecting the actin cytoskeleton of two cells. Gap junctions are also called communicating junctions, since they directly connect the cytoplasm of two neighboring cells. Through this regulated molecular gate, electrical impulses, various molecules, and ions can move directly between the two cells without contacting the extracellular space. Changes in adherens junctions induce an EMT, which leads to stem cell migration, differentiation, and tumor cell genesis [7, 8]. It has been reported that peptidoglycan (PGN), but not LTA, from *S. aureus* can modulate tight junctions [9]. In the current study, we examined whether aLTA isolated from *S. aureus* could induce changes in adherens junctions in HaCaT cells. We showed that a low dose of aLTA induced cell proliferation, whereas a high dose of aLTA induced EMT via changes in adherens junctions, which might lead to the generation of skin tumors.

Materials and Methods

S. aureus Culture

Brain heart infusion (BHI) broth was used to amplify *S. aureus*. Briefly, stock cells were inoculated in 5 ml of BHI broth and incubated overnight at 37°C. Then, 50 µl of cultured cells was sub-cultured in 5 ml of fresh BHI broth and incubated for 18 h at 37°C. Next, 5 ml of cultured cells was transferred to 500 ml of fresh BHI broth and further incubated for 18 h at 37°C. Finally, 500 ml of cultured cells was transferred to 8 L of BHI broth and incubated for 16 h at 37°C.

Purification of aLTA

S. aureus was harvested from the 8 L culture by centrifugation (4,000 ×g, 4°C) and washed three times with distilled water (DW). After centrifugation, the pellet was resuspended in 0.1 M sodium citrate buffer (pH 4.7), and then cells were disrupted by sonication. An equal volume of *n*-butanol was added to the *S. aureus* lysates, and the solution was mixed using a magnetic stirrer for 40 min. After centrifugation at 13,000 ×g for 20 min, the aqueous phase was collected. The aqueous phase containing LTA was dialyzed with DW, and LTA was purified using octyl-Sepharose chromatography. Potential contamination of the isolated LTA was assessed by silver staining for protein contamination and a Limulus amoebocyte lysate test for endotoxin contamination (GenScript, USA). Contamination with nucleic acids was examined by measuring the ratio of absorbance at 260 and 280 nm.

Mammalian Cell Culture and Proliferation Assay

The human keratinocyte cell line HaCaT was maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified 5% CO₂ incubator. For the cell viability assay, HaCaT cells were seeded into 96-well plates, and different doses of aLTA were added to each well. Cells were stimulated for 3 days, and cell viability was determined using a PreMix WST-1 Cell Proliferation Assay System (Takara, Japan). Absorbance was measured using a microplate reader (Eppendorf BioPhotometer, USA) at a test wavelength of 450 nm and reference wavelength of 600 nm.

Real-Time PCR

cDNA was synthesized with total RNA isolated from HaCaT cells treated with aLTA. For total RNA isolation, TRIzol reagent (Takara) was used according to the manufacturer's instructions. An iScript cDNA synthesis kit (Bio-Rad, USA) was used for cDNA synthesis. To determine the expression level of target mRNA, a CFX Connect Real-Time PCR Detection System (Bio-Rad) with a SYBR Ex Taq II master mix (Takara) was used. The following sequences were used for forward and reverse primers: 5'-ATGCTGATGCCCCAATACC-3' and 5'-GCTGTGAGGATGCCAGTTTC-3' for E-cadherin; 5'-TGACAATGACCCACAGCTC-3' and 5'-GTCCTGCTCACCACCACTAC-3' for N-cadherin; 5'-CGAGTGGTCTTCTGCGCTA-3' and 5'-TGCAGCTCGCTGTAGTTAGG-3' for Snail; and 5'-GTCTTACCACCATGGAGAA-3' and 5'-AGGAGGCATTGCTGATGATC-3' for GAPDH. To inhibit the appropriate signaling pathways, cells were treated with NF-κB (#481406) and extracellular signal-regulated protein kinase (ERK) (#328006) inhibitors (Merck, Kenilworth, USA) followed by aLTA. mRNA expression was examined by real-time PCR. Gene expression was normalized to GAPDH.

Western Blot Analysis

aLTA-treated HaCaT cells were lysed with 2× Laemmli sample buffer. After boiling at 100°C for 5 min, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on an 8–12% gel and transferred onto a polyvinylidene fluoride membrane. Then, 5% skim milk dissolved in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20) was used as the blocking solution. The primary antibodies for phospho-ERK1/2, phospho-NFκB-p65, phospho-SAPK/JNK, phospho-p38, phospho-cAMP response element binding protein (CREB) (Cell Signaling Technology, USA), E-cadherin, N-cadherin, and β-actin HRP (Santa Cruz, USA) were diluted in TBS-T and incubated with the membranes overnight at 4°C. HRP-conjugated secondary antibodies for mouse or rabbit (Santa Cruz) were diluted in TBS-T and incubated with the membranes for 1 h at room temperature. Protein bands were detected by enhanced chemiluminescence reagent and with β-actin as a loading control.

Wound Healing Assay

HaCaT cells were seeded into a 6-well plate at approximately

80% confluency. When cells were 100% confluent, a wound in the culture was made using a pipette tip. After washing to remove debris and the culture medium, fresh medium was added to the culture. Before stimulation with aLTA, a phase-contrast microscopic image of the cells was taken. Another picture was taken after 24 h of stimulation with aLTA. The healed area was calculated using ImageJ software.

Statistical Analysis

The data are shown as the means $\pm$ SDs of at least three experiments. Differences were considered statistically significant when the p -value was <0.05 .

Results

High Doses of aLTA Promoted Phenotypic Changes of HaCaT Cells into Mesenchymal-Like Cells

Similarly to other cell wall components, such as PGN, teichoic acid, and lipopolysaccharide, aLTA induces proinflammatory cytokine production from macrophages and also has cytotoxic activity [10]. To examine the cytotoxicity of aLTA in HaCaT cells, cells were treated with varying doses of aLTA for 24, 48, and 72 h, and cell viability was examined with a WST-1 cell proliferation assay. The population of HaCaT cells treated with 1 and 10 $\mu\text{g}/\text{ml}$ of aLTA increased and survived until 72 h. On the other hand, 100 $\mu\text{g}/\text{ml}$ of aLTA decreased the cell population when compared with controls, indicating that the high dose of aLTA induced cell death after 48 h of treatment (Fig. 1A).

In addition, aLTA treatment increased phenotypic changes in HaCaT cells in an aLTA dose dependent manner. As shown in Fig. 1B, some of the cells stimulated by aLTA showed a mesenchymal phenotype, suggesting that aLTA induced EMT in HaCaT cells.

High Doses of aLTA Upregulated Mesenchymal Phenotype Markers in HaCaT Cells

The downregulation of E-cadherin in epithelial cells is considered to be a primary event in EMT. On the other hand, the expression of N-cadherin and Snail-1 is increased when the transition is complete [6]. In the current study, when HaCaT cells were treated with 1 $\mu\text{g}/\text{ml}$ of aLTA, the mRNA level of E-cadherin was increased up to 30-fold compared with controls after 12 h of stimulation. This mRNA level was decreased by 3.2-fold in cells treated with 10 $\mu\text{g}/\text{ml}$ aLTA. Interestingly, E-cadherin mRNA expression levels decreased over 50% in cells treated with 100 $\mu\text{g}/\text{ml}$ of aLTA compared with untreated cells (Fig. 2A). On the other hand, the mRNA levels of N-cadherin and Snail-1 were unchanged and both decreased about 50% after treatment with 1 $\mu\text{g}/\text{ml}$ of aLTA. Importantly, mRNA levels of N-cadherin and Snail-1 increased 1.5- and 4-fold, respectively, after treatment with a dose higher than 10 $\mu\text{g}/\text{ml}$ of aLTA. When cells were treated with 100 $\mu\text{g}/\text{ml}$ of aLTA, the mRNA levels of both N-cadherin and Snail-1 were increased by about 8-fold (Fig. 2B). By contrast, the protein level of E-cadherin was increased 3 h after 1 $\mu\text{g}/\text{ml}$ of

Fig. 1. aLTA differentially affects HaCaT cell viability.

(A) HaCaT cells were treated with aLTA at the indicated concentrations and times in 96-well plates. Cell viability was assessed with WST-1 reagent. (B) Morphological changes in HaCaT cells treated with 0, 1, 10, and 100 $\mu\text{g}/\text{ml}$ aLTA were observed under a microscope. Data are presented as the mean $\pm$ SD; * $p < 0.05$, *** $p < 0.001$ compared with untreated cells. Red arrows indicate cells having mesenchymal phenotype.

Fig. 2. aLTA regulated mesenchymal phenotype marker expression.

(A) E-Cadherin mRNA expression was increased in HaCaT cells treated with 1 µg/ml aLTA for 12 h. (B) N-Cadherin and Snail-1 mRNA expression was increased after treatment with both 10 and 100 µg/ml aLTA. (C) E-Cadherin and N-cadherin protein expression levels were examined by western blot after aLTA treatment. Densitometry analysis for E-cadherin (D) and N-cadherin (E) protein band intensities was done by ImageJ software after repeated experiments. Data are presented as the mean $\pm$ SD; ** p < 0.01, *** p < 0.001 compared with untreated cells.

aLTA treatment, and this expression was maintained up to 24 h after treatment. However, E-cadherin in cells treated with 100 µg/ml aLTA was decreased at the protein level after 12 h of stimulation. On the other hand, the N-cadherin protein level was upregulated by 100 µg/ml of aLTA at 12 h, whereas expression of this protein was not increased in cells stimulated with 1 or 10 µg/ml aLTA (Fig. 2C). The densitometry results for E-cadherin (Fig. 2D) and N-cadherin (Fig. 2E) was added. These results indicated that a low dose of aLTA increased E-cadherin expression, whereas a high dose of aLTA increased N-cadherin and Snail-1 expression. This gene expression pattern is a hallmark of EMT, and evidence of the mesenchymal transition of the HaCaT cells treated with high doses of aLTA.

EMT in HaCaT Cells Treated with a High Dose of aLTA Was Mediated by the Inactivation of NF- κ B and by the Activation of ERK1/2

To examine the effect of diverse doses of aLTA on the TLR-mediated signaling pathway, the expression levels of certain MAPKs, such as ERK1/2, JNK1/2, and p38, Akt,

and transcription factors, such as CREB and NF- κ B, were examined. When cells were stimulated with 1 µg/ml of aLTA, phosphorylation of the NF- κ B p65 subunit was increased (Fig. 3A, left panel). This activation of NF- κ B was not shown in the cells stimulated with 10 and 100 µg/ml aLTA. Instead, both 10 and 100 µg/ml aLTA increased the phosphorylation of ERK1/2 (Fig. 3A, middle and right panels). The activation of ERK1/2 was not shown in 1 µg/ml aLTA-treated cells. From these results, we postulated that E-cadherin was upregulated by NF- κ B, which was itself activated by 1 µg/ml aLTA, whereas N-cadherin and Snail-1 were upregulated by ERK1/2, which were activated by 10 or 100 µg/ml aLTA. The activation of JNK, Akt, and CREB was not altered significantly in the time points that we examined. To confirm the role of NF- κ B and ERK1/2 in adherens junction-related gene expression, cells were treated with either an NF- κ B or ERK1/2 inhibitor, followed by treatment with aLTA. As we expected, the upregulation of E-cadherin seen in 1 µg/ml aLTA-treated cells was inhibited by the NF- κ B inhibitor, whereas N-cadherin and Snail-1 expression levels were inhibited by the ERK1/2

Fig. 3. NF- κ B and ERK1/2 activation was induced by aLTA.

(A) HaCaT cells were treated with aLTA at the indicated concentrations and times, and then the activation of several signaling molecules was examined by western blot analysis. (B) The mRNA expression of mesenchymal markers was examined by real-time PCR after treatment with inhibitors followed by treatment with either 1 or 100 μ g/ml aLTA. Data are presented as the mean $\pm$ SD; *** p < 0.001 compared with untreated cells.

inhibitor (Fig. 3B), suggesting that adherens junction-related gene expression is differentially regulated by low or high doses of aLTA. Taken together, these results suggested that EMT seen in the 100 μ g/ml aLTA-treated cells was mediated by the combined inactivation of NF- κ B and activation of ERK1/2.

Low Dose of aLTA Induced the Proliferation of HaCaT Cells

In Fig. 1A, we show that a low dose of aLTA induced cell proliferation in a time-dependent manner. The proliferation of aLTA-treated HaCaT cells was assessed using a wound healing assay. Images were taken just after the wound was made, and after 24 h of aLTA treatment. When HaCaT cells were treated with 1 μ g/ml of aLTA, the rate of wound

healing was increased when compared with untreated cells. A similar healing rate was shown in cells treated with 10 μ g/ml of aLTA. However, when cells were stimulated with 100 μ g/ml of aLTA, the rate of healing was lower than that of the control cells (Fig. 4A). ImageJ software was used to calculate the healing rate (Fig. 4B). These results suggested that a low dose of aLTA induced HaCaT cell proliferation, which resulted in wound healing.

Discussion

Immune cells secrete pro- or anti-inflammatory cytokines through the TLR-mediated signaling pathway when they encounter bacteria. TLR-mediated cytokine production regulates both innate and adaptive immunities. It is well

Fig. 4. Low-dose aLTA induced proliferative activity of HaCaT cells.

(A) HaCaT cells were treated with various doses of aLTA for 24 h. Images were taken at 0 and 24 h. (B) Comparative chart of the healed area rates. Data are presented as the mean $\pm$ SD; * p < 0.05, ** p < 0.01 compared with untreated cells.

known that LTA is recognized by TLR2/TLR6, and activates NF- κ B and/or MAPKs [11]. For example, aLTA isolated from *S. aureus*, sLTA isolated from *L. sakei*, and dLTA isolated from *L. delbrueckii* strongly induce TNF- α production [3, 12]. pLTA isolated from *L. plantarum*, however, did not induce inflammatory responses, but instead inhibited proinflammatory cytokine production in cells stimulated by endotoxins [12]. Although every isoform of LTA is recognized by a common receptor, TLR2, the response of immune cells to different forms of LTA varies, due to the distinct structures of the isoforms. In this study, we examined the effect of aLTA on the regulation of adherens junction proteins in HaCaT cells. Previously, Kuo *et al.* [9] showed that PGN from *S. aureus* enhanced tight junctions, whereas LTA did not. In the current study, we also observed that aLTA only moderately induced or reduced tight junction-related protein expression (data not shown), whereas it significantly regulated adherens junction-related protein expression. When HaCaT cells were treated with 100 μ g/ml aLTA, cell proliferation was reduced and EMT was induced. In the other study, we observed that CREB3L1 and VEGF levels were changed by aLTA and the cell population was significantly decreased after 48 h (data not shown). Moreover, 100 μ g/ml aLTA also increased caspase activity and decreased the Bcl2 level, which led to cell apoptosis. Thus, a high dose of aLTA reduces HaCaT cell population through apoptosis, but survived cells then go to the EMT state. As we addressed in Figs. 2 and 3, E-cadherin and N-cadherin/Snail-1 are involved in the cell proliferation or wound healing and EMT of HaCaT cells. Wound healing and EMT might be regulated by signaling pathways, including ERK and NF- κ B, which are differently regulated by a low and high dose of aLTA. The downregulation of E-cadherin and upregulation of N-cadherin and Snail-1 were observed in cells treated with a high dose of aLTA, which activated the ERK signaling pathway. Activation of ERK signaling has been shown to inhibit the degradation of Snail-1 in tumor cells [13]. When cells were given a low dose of aLTA, NF- κ B was activated, leading to an increase in E-cadherin expression. In addition, a low dose of aLTA enhanced proliferation of HaCaT cells. On the other hand, a high dose of aLTA increased EMT. Although cell viability was decreased after 24 h of treatment with a high dose of aLTA, the surviving cells exhibited a mesenchymal phenotype, indicating that they had undergone EMT.

During the wound healing process, cell proliferation and reconstruction of the extracellular matrix are both increased to maintain cell and tissue structure. Thus, the effect of procollagen and matrix metalloproteinase (MMP) modulated

by low-dose aLTA in keratinocytes should be addressed [14]. In the cells treated with a high dose of aLTA, EMT was induced, which may eventually promote the generation of tumors instead of wound healing. In future studies, the role of MMP expression, and cell invasion and migration caused by high doses of aLTA should be elucidated [15]. It is known that LTA induces EMT-mediated fibrosis through binding to TLRs [16]. In addition, because certain mutations in TLRs are known to induce tumor cell genesis, a study investigating the aLTA-TLR2-mediated signaling pathway would be useful to identify the underlying mechanisms of *S. aureus* infection and tumor cell genesis in keratinocytes [17, 18].

In this study, the interaction between HaCaT cells and aLTA was shown to vary in a dose-dependent manner. This suggests that the presence of *S. aureus* may induce either tumor cell genesis and EMT via adherens junction changes, or wound healing via immune activation in the infected region according to the population density of the bacteria. Monophosphoryl lipid A is a derivative of lipid A isolated from *Salmonella minnestora* R595 lipopolysaccharide [19, 20]. As a TLR4 agonist, it has been used as a vaccine adjuvant [21]. Similarly, aLTA could be considered as an agent to promote wound healing, although it should be modified to reduce toxicity against host cells.

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