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Staphylococcus aureus Infection of Epidermal Keratinocytes Promotes Expression of Innate Antimicrobial Peptides

Barbara E. Menzies[1,2]* and Aimee Kenoyer[1]

Medical Research Service of the Veterans Affairs Puget Sound Health Care System, Seattle, Washington,[1] and Division of Allergy and Infectious Diseases, University of Washington, Seattle, Washington[2]

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Keratinocytes upregulate expression of endogenous antimicrobial peptides in response to inflammatory stimuli. We show that both viable and heat-inactivated *Staphylococcus aureus* and lipoteichoic acid differentially alter expression of these peptides upon contact with human keratinocytes. The findings indicate a diversity of staphylococcal factors involved in upregulation of antimicrobial peptide expression in cutaneous epithelia.

Skin epithelium provides a mechanical barrier to invading pathogens and also participates in innate immune defense by producing cationic antimicrobial peptides that inactivate a wide variety of microorganisms. In human skin epithelia, the two main antimicrobial peptide families are the β-defensins (hBD) (6) and the cathelicidin LL-37 (15). With the possible exception of hBD-1, peptide expression is inducible by various inflammatory stimuli, including cytokines and microbial products (2, 3, 4, 7, 8).

Staphylococcus aureus is a common cutaneous commensal and pathogen. Heat-inactivated *S. aureus* induces increased expression of hBD-2, hBD-3, and LL-37 in primary keratinocytes (12). The bacterial factors that play a role in staphylococcal induction of these peptides are not well elucidated. In this report, we compare the coordinate patterns of gene expression of hBD-1, hBD-2, hBD-3, and LL-37 in keratinocytes challenged with either live or heat-inactivated *S. aureus*. We also examine the bacterial components and host-pathogen interactions that are critical for expression of these endogenous peptide antibiotics.

Human neonatal keratinocytes were cultivated in serum-free keratinocyte growth medium (Clonetics) supplemented with bovine pituitary extract, epidermal growth factor, insulin, and hydrocortisone at 37°C in a 5% CO_2 incubator. At 80% confluence, the cells were shifted from media with 0.15 mM Ca^{2+} to media containing higher Ca^{2+} concentrations (1.15 mM) and maintained there for 3 to 4 days to induce differentiation. Addition of calcium is commonly used to induce a differentiated state in which cells become stratified and express differentiation markers similar to cells in the upper epidermis (14). To examine host cell-bacterial interactions in this model, differentiated keratinocytes were coincubated with viable *S. aureus* strain DK2076, a clinical isolate that is methicillin susceptible. The keratinocytes were infected at a multiplicity of infection (MOI) of 50 to 100 for 1 h and then processed for transmission electron microscopy (TEM). Staphylococci bound primarily to filopodia or membranous protrusions of the keratinocytes (Fig. 1A) and in some instances appeared to adhere tightly by pilus-like structures within electron-dense grooves along the keratinocytes (Fig. 1B). In some cells, staphylococci resided intracellularly within membrane-bound vacuoles (Fig. 1C). Thus, TEM confirmed specific adherence and uptake of *S. aureus* within this Ca^{2+}-differentiated keratinocyte cell model, similar to those shown with undifferentiated proliferating cells (10).

Enhanced expression of hBD-2 in keratinocytes upon Ca^{2+}-induced differentiation has previously been demonstrated (5, 7). We examined the effect of Ca^{2+} differentiation upon basal mRNA levels of hBD-1, hBD-3, and LL-37, in addition to hBD-2. Total RNA harvested from treated keratinocytes was reverse transcribed and used as a template for quantitative real-time PCR, using SYBR green I dye as the amplicon detector and ROX (5-carboxy-X-rhodamine) as the passive reference. The gene for β-actin was amplified as an endogenous reference. Quantification was done using both the standard-curve and the comparative threshold cycle methods. Primers were designed with the aid of Primer Express software (Applied Biosystems, Tokyo, Japan); primer pairs are listed in Table 1. Results are expressed as mean values ± standard deviations from at least three independent experiments. Student's *t* test was performed in Microsoft Excel, and differences were considered statistically significant at P values of <0.05. We confirmed that numbers of basal hBD-2 transcripts were significantly increased in cells differentiated with increased Ca^{2+} compared to those in cells with low Ca^{2+} (Fig. 2). The numbers of transcripts of hBD-1 and LL-37 increased to a lesser degree (approximately twofold); however, those of hBD-3 were not appreciably affected. Thus, our results corroborate the upregulated gene expression of hBD-2 and additionally demonstrate increased levels of basal mRNA transcripts of hBD-1 and LL-37 in Ca^{2+}-differentiated cells. All subsequent experiments were performed using Ca^{2+}-differentiated cells.

We next investigated whether exposure to live *S. aureus* organisms could modulate the expression of these antimicrobial peptides in Ca^{2+}-differentiated keratinocytes. Cells were coincubated with viable *S. aureus* at an MOI of 50 to 100, and following specified time periods, total RNA was extracted for real-time PCR analysis for gene expression. Live staphylococcal organisms induced significant increases in gene expression of defensins hBD-1 and hBD-3 in keratinocytes after 1.5 h of coincubation (Fig. 3A). hBD-2 and LL-37 mRNA levels were

* Corresponding author. Mailing address: Veterans Affairs Puget Sound Health Care System, S-111-ID, 1660 S. Columbian Way, Seattle, WA 98108. Phone: (206) 277-4576. Fax: (206) 764-2689. E-mail: bmenzies@u.washington.edu.

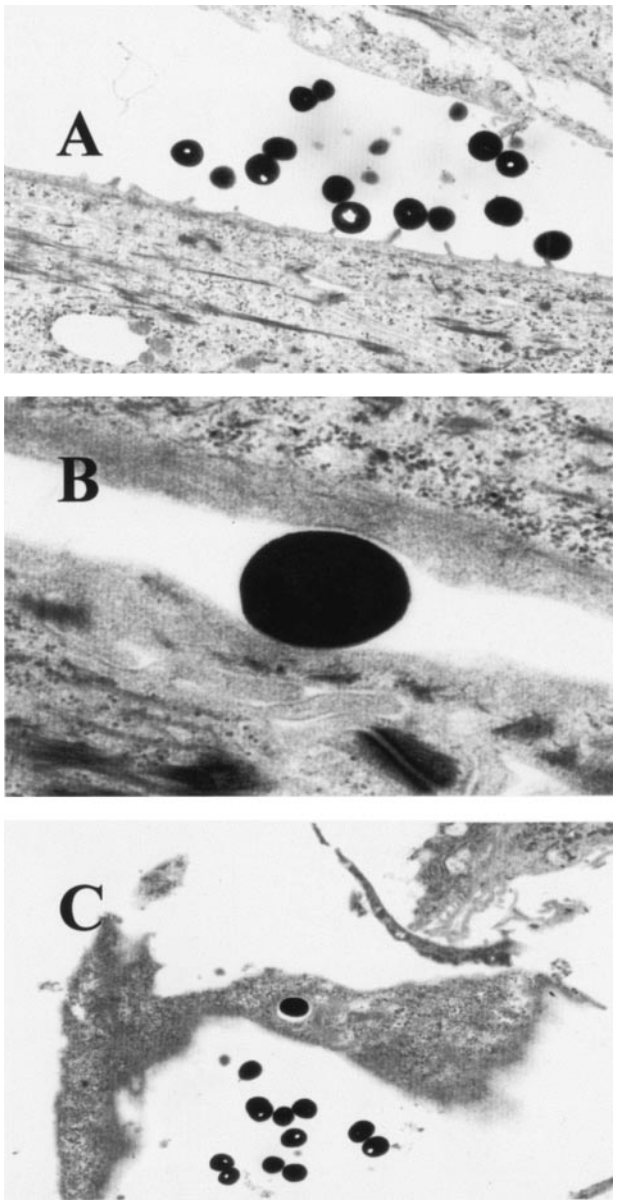

FIG. 1. Transmission electron micrographs revealing ultrastructural interactions of viable *S. aureus* organisms upon contact with differentiated keratinocytes. (A) Staphylococci bind to filopodia-like extrusions of the host cell membrane (magnification, ×6,000). (B) At higher magnification, staphylococci adhere within an electron-dense groove along the membrane (magnification, ×25,000). (C) A few keratinocytes contained staphylococci within vacuoles (magnification, ×30,000).

not significantly increased in response to viable staphylococci after 1.5 h.

We then sought to determine the effect of intracellular staphylococci upon antimicrobial peptide gene induction. As demonstrated above with TEM, some staphylococci are internalized within intracellular vacuoles in as little as 30 min. After a 2-h coincubation of keratinocytes with staphylococci, the keratinocytes were treated briefly with lysostaphin to kill extracellular staphylococci and the culture medium was replaced with fresh medium containing lysostaphin. This treatment does not affect internalized staphylococci, which remain viable during the course of the assay (data not shown). Gene expression of antimicrobial peptides in keratinocytes harboring intracellular *S. aureus* organisms over a more prolonged period was then examined. No increase in transcript levels was detectable at 5 h; however, at 24 h of intracellular staphylococcal infection, the mRNA levels of hBD-1, hBD-2, and hBD-3 were strongly upregulated, with increased expression most marked for hBD-2 and hBD-3 (Fig. 3B). In comparison, the LL-37 transcript was not significantly increased at 24 h. The results suggest that the presence of *S. aureus* within the intracytosolic compartment of keratinocytes can contribute to enhanced hBD-1, hBD-2, and hBD-3 gene expression.

We next compared the gene expression of antimicrobial peptides in keratinocytes exposed to heat-killed staphylococci to that in cells treated with viable staphylococci. Although heat

TABLE 1. Primer pairs

Gene	Direction of transcription	Sequence
hBD-1	Forward	5′-ACA ATT GCG TCA GCA GTG GAG G-3′
	Reverse	5′-TCA CTT GCA GCA CTT GGC CTT C-3′
hBD-2	Forward	5′-CAC CTG TGG TCT CCC TGG AAC A-3′
	Reverse	5′-GCC CTT TCT GAA TCC GCA TCA-3′
hBD-3	Forward	5′-AAG CCT AGC AGC TAT GAG GAT CC-3′
	Reverse	3′-TGT GTT TAT GAT TCC TCC ATG ACC-3′
LL-37	Forward	5′-CCG GAA ATC TAA AGA GAA GAT TGG-3′
	Reverse	5′-CAC ACT AGG ACT CTG TCC TGG GTA-3′
β-Actin	Forward	5′-ATC GTC ACC AAC TGG GAC GAC A-3′
	Reverse	5′-ATC TGG GTC ATC TTC TCG CGG T-3′

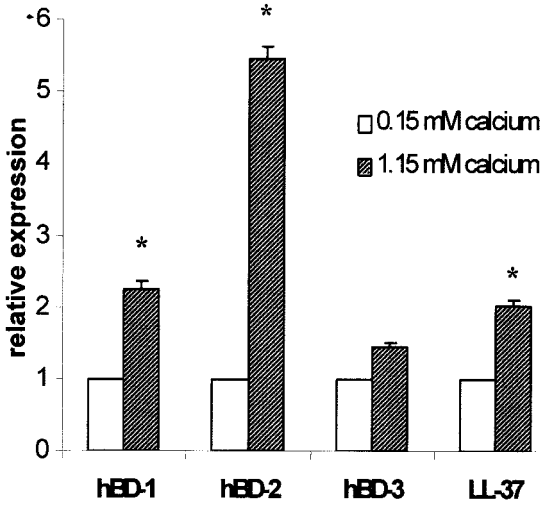

FIG. 2. Relative levels of expression of hBD-1, hBD-2, and cathelicidin LL-37 mRNA are dependent upon the exposure of keratinocytes to an elevated calcium concentration. Keratinocytes were grown to 80% confluence and then propagated further with media containing either 0.15 mM calcium or 1.15 mM calcium for 4 days. Total RNA was harvested for reverse transcription, and the cDNA was used in a real-time PCR analysis. The results of real-time analysis are expressed as ratios in comparison to the values obtained from keratinocytes in low-calcium medium and are means ± standard deviations. *, $P < 0.05$.

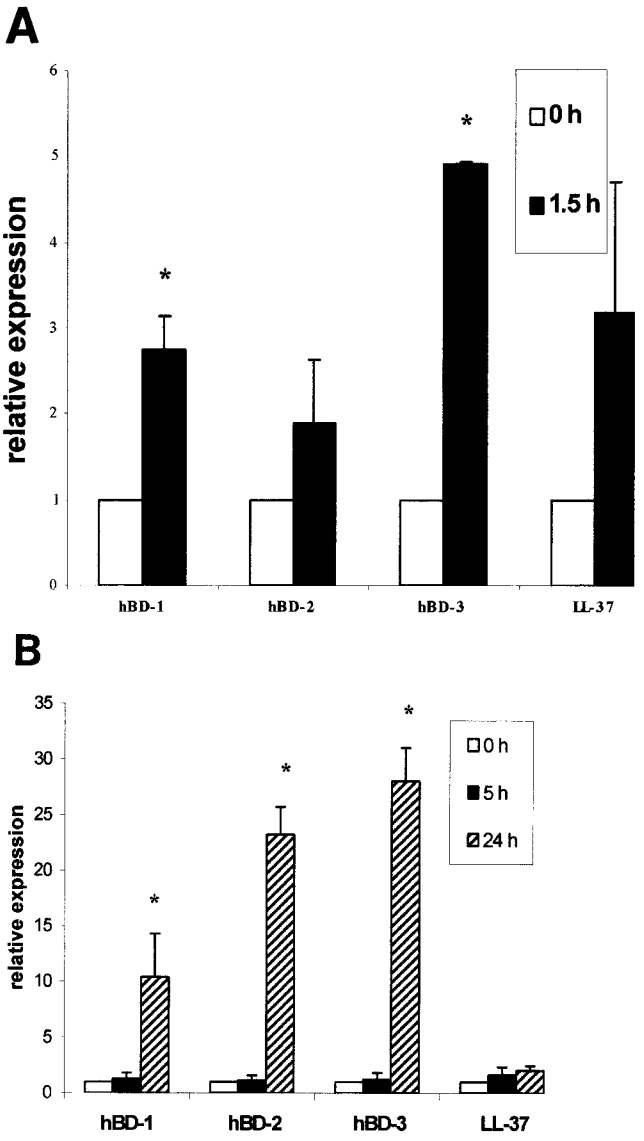

FIG. 3. Relative levels of expression of hBD-1, hBD-2, hBD-3, and LL-37 mRNA in Ca^{2+}-differentiated keratinocytes challenged with live *S. aureus* for 1.5 h (A). After 2 h of coincubation, some wells were treated with lysostaphin to kill extracellular staphylococci, and then the medium was replaced with fresh medium containing lysostaphin for incubation up to 24 h (B). The results of real-time analysis are expressed as ratios in comparison to the values at zero time and are means ± standard deviations. *, $P < 0.05$.

inactivation of bacteria does not permit study of host-pathogen interactions as influenced by secreted bacterial molecules and heat-sensitive cell wall components, it does allow investigation into the role of heat-stable bacterial cell wall constituents in host responses. Despite use of a similar MOI of ~50, exposure of Ca^{2+}-differentiated keratinocytes to washed heat-killed *S. aureus* organisms failed to show the early increased gene expression of cationic antimicrobial peptides noted above for keratinocytes infected with viable staphylococci. Unlike the inducible hBD-1 transcript observed with stimulation by live staphylococcal organisms, heat-killed staphylococci did not trigger an increased hBD-1 message (Fig. 4). However, the gene expression

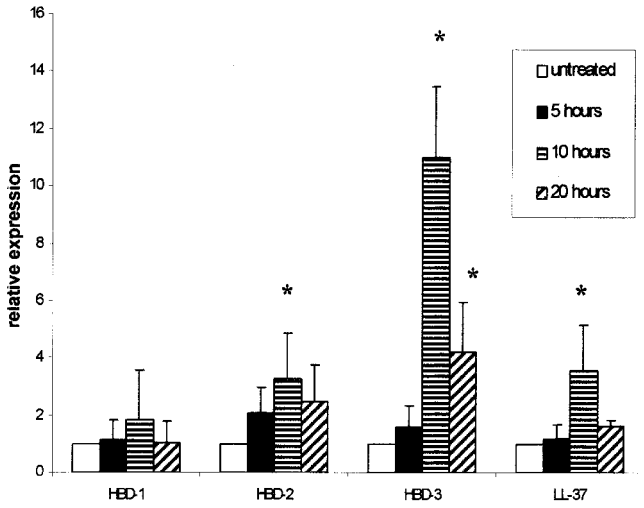

FIG. 4. Relative levels of expression of hBD-1, hBD-2, hBD-3, and cathelicidin LL-37 mRNA in Ca^{2+}-differentiated keratinocytes stimulated with heat-killed *S. aureus* for up to 20 h. Total RNA was extracted for real-time PCR. Untreated cells were harvested for the zero-hour sampling time. The results are expressed as ratios in comparison to the values at zero time and are means ± standard deviations. *, $P < 0.05$.

of hBD-2, hBD-3, and LL-37 was significantly upregulated by exposure to heat-killed *S. aureus*, peaking at 10 h and then dropping toward baseline by 20 h. Therefore, the results corroborate those of an earlier study (12) and suggest that *S. aureus* possesses one or more heat-stable cell wall components that trigger enhanced expression of these peptide genes in keratinocytes.

We next investigated whether lipoteichoic acid (LTA), a major constituent in the cell wall of *S. aureus*, was capable of eliciting mRNA induction of cationic antimicrobial peptide genes. Highly purified and endotoxin-free LTA ($<3 \times 10^{-3}$ endotoxin units/ml, as determined by a *Limulus* amoebocyte lysate assay) was applied to differentiated keratinocyte cultures

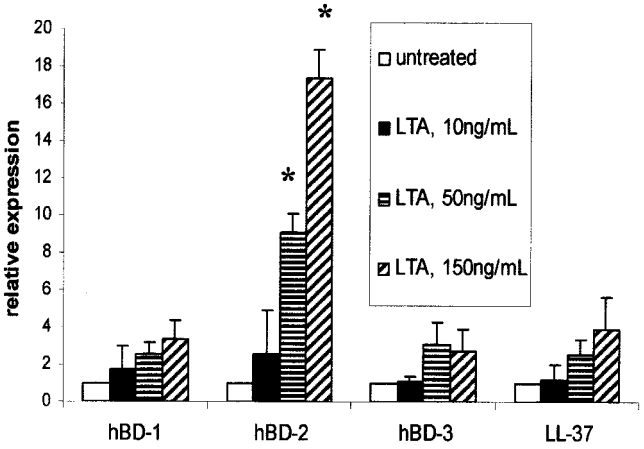

FIG. 5. Dose response of the effect of *S. aureus* LTA on the expression of antimicrobial peptides in Ca^{2+}-differentiated keratinocytes. Cells were treated with various concentrations of LTA for 5 h, and RNA was harvested for reverse transcription and real-time PCR analysis. The results are expressed as ratios in comparison to the values of untreated cells and are means ± standard deviations. *, $P < 0.05$.

at concentrations up to150 ng/ml. LTA treatment of keratinocytes for 5 h strongly stimulated hBD-2 transcripts in a concentration-dependent manner (Fig. 5). There was a tendency toward increased transcription of hBD-3 and LL-37 genes in response to LTA, but this was not statistically significant.

We have demonstrated that both viable and heat-inactivated *S. aureus* isolates differentially alter the expression of endogenous antimicrobial peptides upon contact with keratinocytes. Live *S. aureus* organisms induced both an early (1.5 h) and a late (24 h) upregulation of both hBD-1 and hBD-3 gene expression. On the other hand, heat-inactivated organisms stimulated upregulation of all studied genes except for that encoding hBD-1; this constitutive expression of hBD-1 is consistent with that observed with other microbial stimuli. The differential expression highlights the complexities in analyzing host responses when only inactivated bacteria are used for stimulation. Use of killed staphylococci does not permit assessment of the full repertoire of bacterial products delivered by this pathogen.

Application of lysostaphin to the coculture to remove extracellular staphylococci permitted observation of keratinocyte responses to internalized staphylococci over a more extended period. At 24 h, a more pronounced upregulation of hBD-1, hBD-2, and hBD-3 gene transcripts was observed. *S. aureus* is capable of secreting a huge arsenal of virulence determinants that are known to elicit the expression of a number of inflammatory cytokines and other molecules (9), and thus, these microbial factors might also stimulate the expression of host antimicrobial peptides. Alternatively, in this cell culture model, the peptide genes may also be upregulated in an autocrine or paracrine manner by production of interleukin-1α/β or interleukin-6 from *S. aureus*-infected keratinocytes. Lysostaphin in this model is effective in inhibiting the growth of any released intracellular staphylococci; nonetheless, we cannot entirely exclude the stimulatory action of staphylococcal cell wall products from the lysostaphin-induced cell wall lysis of any released bacteria.

The induction of hBD-2, hBD-3, and LL-37 in keratinocytes exposed to heat-inactivated *S. aureus* suggested that one or more heat-stable microbial cell wall constituents contribute to this host cell response. Indeed, highly purified and endotoxin-free LTA stimulated hBD-2, hBD-3, and LL-37 mRNA in a dose-dependent manner, although this appeared to be significant for hBD-2 gene expression only. We cannot exclude the possibility of inducible effects from peptidoglycan, another major staphylococcal cell wall component. Both LTA and peptidoglycan are ligands for Toll-like receptor 2 (TLR-2), which is part of a family of innate immune recognition receptors involved in pattern recognition of microbial pathogens (13). TLR-2 receptors in myeloid cells have been best characterized, but functionally active TLR-2 is also expressed in epithelial cells, including keratinocytes (11). Activation of TLR-2 signaling leads to downstream translocation of the proinflammatory transcription factor nuclear factor-κB (NF-κB) and enhanced transcription of NF-κB-controlled genes, such as those for cytokines and chemokines. The hBD-2 gene promoter contains several consensus sequence-binding sites for NF-κB transcription factors (1). We speculate that LTA binds to TLR-2, signaling the activation of NF-κB gene transcription for the downstream gene expression of hBD-2.

All together, these findings suggest that *S. aureus* is endowed with a diverse array of microbial components that may signal enhanced peptide gene expression from the extracellular or intracellular compartment. Studies are under way to identify staphylococcal determinants other than LTA or other host interactions that are involved in the induction of these critical components of the innate immune system.

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