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The *in vitro* immune-modulating properties of a sweat gland-derived anti-microbial peptide dermcidin

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Abstract

The epidermal barriers of the skin serve as the first layer of defense by limiting the access of many pathogens to the blood circulation. In addition, human skin also contains sweat glands that can secrete a wide array of antimicrobial peptides to restrain the growth of various microbes. In the case of microbial infection, macrophages and monocytes constitute of the first line of defense by producing a wide array of proinflammatory cytokines and chemokines. This process is triggered either by pathogen-associated molecular pattern molecules (PAMPs, such as bacterial endotoxin) or damage-associated molecular pattern molecules (DAMPs such as HMGB1). In light of our findings that a sweat gland-derived antimicrobial peptide, dermcidin, affected both PAMP- and DAMP-induced cytokines/chemokines by macrophages/monocytes, we propose that dermcidin may play an important role in the regulation of the innate immune responses to infection and injury. Future investigations are warranted to further test this understudied hypothesis in both preclinical and clinical settings.

Introduction

Cohabiting with various microbes over millions of years, animals have developed multiple strategies to deal with microbial infections. The epidermal barriers of the skin serve as the first layer of defense by limiting the physical access of many pathogens to the blood circulation. In addition, sweat glands secrete a wide array of antimicrobial peptides, which restrain the growth of various microbes on the skin. For instance, during rigorous physical exercise, an antimicrobial peptide, called dermcidin, is secreted by the sweat glands onto the epidermal surface of the skin (1). It has been proposed that dermcidin can be activated in salty and slightly acidic sweat to form channels that can possibly perforate microbe membranes, allowing water and charged Zn^{+2} ions in sweat to gush across the cell membrane, killing the microbe (2, 3). Despite its capacity in binding to various bacterial strains, dermcidin has not yet been reproducibly shown to permeabilize bacterial membranes (4), calling for further investigation in this arena. Nevertheless, at body sites in frequent

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Author contributions

X.Q., S.Z., P.W., designed the study; E.W., X.Q., and S.Z. performed the experiments; J. L. provided expert assistance with the preparation of recombinant proteins; E. W., X.Q., S.Z. and P.W. analyzed data; E. W. and P. W. wrote the manuscript.

contact with pathogenic microbes, a higher amount of dermcidin peptide is detected in sweat (5), supporting the essential role of sweat in the regulation of skin microbial flora. If the skin epithelial barrier is breached, the host's innate immune system immediately mounts a biological response, termed "inflammation", to confine and remove the invading pathogens (6). In case of severe injury and infection, the invading pathogens can leak into the blood stream, triggering widespread and systemic inflammatory responses. It was previously unknown whether antimicrobial agents such as dermcidin could also exhibit immune-modulating properties in response to infection or injury.

As the first line of defense against microbial infection, monocytes continuously patrol the body in search of invading pathogens or damaged tissues, and can immediately infiltrate the infected/injured tissue upon the detection of microbial products or host-derived chemotactic factors. Once reaching extravascular tissues, these monocytes are differentiated into tissue-specific resident macrophages, which ingest and eliminate invading pathogens in conjunction with other phagocytes (e.g., neutrophils). Additionally, macrophages/monocytes are equipped with pattern recognition receptors [such as the Toll-like receptors (TLRs) TLR2, TLR3, TLR4, and TLR9] (7) for various pathogen-associated molecular patterns (PAMPs, such as bacterial peptidoglycan, double-stranded RNA, endotoxin, and CpG-DNA) (8). The engagement of various PAMPs with respective receptors triggers release of various proinflammatory mediators such as high mobility group box 1 (HMGB1) (9), cold-inducible RNA-binding protein (CIRP) (10, 11) as well as nitric oxide (NO) (12). In addition to active secretion, HMGB1 can also be passively released from damaged cells (13) following ischemia/reperfusion (14), trauma (15), or toxemia (16), thereby serving as a damage-associated molecular pattern molecule (DAMP). Thus, infection and injury converge on a common process, inflammation (17), which is orchestrated by HMGB1 and other proinflammatory mediators (e.g., CIRP) derived from activated immune cells and damaged tissues (10). If dysregulated, the excessive production of these proinflammatory mediators (e.g., HMGB1, NO, and CIRP) (9, 10, 12, 18), individually or in combination, contribute to the pathogenesis of inflammatory diseases.

As aforementioned, dermcidin can be secreted by the sweat glands onto the epidermal surface of the skin (1), and potentially restrains the proliferation of skin microbial flora (2, 3). Here we provide emerging evidence to support an understudied hypothesis that dermcidin exhibits immune-modulating properties in response to PAMP or DAMP.

Materials and Methods

Materials

Bacterial endotoxin (lipopolysaccharide, LPS, *E. coli* 0111:B4, Cat. No. L4130) was obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM, Cat. No. 11995-065), penicillin/streptomycin (Cat. No. 15140-122) and fetal bovine serum (FBS, Cat. No. 26140079) were from Invitrogen (Grand Island, New York). Recombinant HMGB1 and CIRP were expressed in *E. coli*, and purified to remove contaminating endotoxin by Triton X-114 extraction as previously described (10, 19). To express recombinant dermcidin, the cDNA encoding for the mature form of dermcidin (DCD, NM_053283.2) (corresponding to residues 20–110, without the N-terminal signal

peptide, amino acid 1–19) was cloned onto a pReceiver-B01 (CS-T3198-B01-01, GeneCopoeia) vector, and the recombinant DCD was expressed in *E. coli* BL21 (DE3) pLysS cells. Recombinant DCD containing an N-terminal histidine tag (His-DCD) was isolated and purified to remove contaminating endotoxin by Triton X-114 extraction.

Cell culture

Murine macrophage-like RAW 264.7 were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and were cultured in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS. Human blood was purchased from the Long Island Blood Bank (Melville, NY), and human peripheral blood mononuclear cells (HuPBMCs) were isolated by density gradient centrifugation through Ficoll (Ficoll-Paque PLUS, Pharmacia, Piscataway, NJ) as previously described (20–22). Adherent macrophages or HuPBMCs were gently washed with, and cultured in, DMEM before stimulation with LPS (0.4 µg/ml), CIRP (2.0 µg/ml), or HMGB1 (1.0 µg/ml) in the absence or presence of recombinant dermcidin for 16 h. Subsequently, the cell-conditioned culture media were analyzed respectively for levels of nitric oxide, and other cytokines by the Griess Reaction and Cytokine Antibodies Arrays as previously described (19, 23).

Nitric oxide (NO) assay

The levels of NO in the culture medium were determined indirectly by measuring the NO²⁻ production with a colorimetric assay based on the Griess reaction (21, 24). NO²⁻ concentrations were determined with reference to a standard curve generated with sodium nitrite at various dilutions.

Cytokine antibody array

Human Cytokine Antibody Array C3 (Cat. No. AAH-CYT-3-4, RayBiotech Inc., Norcross, GA, USA), which respectively detect 42 cytokines on one membrane, were used to determine cytokine levels in human monocyte-conditioned culture medium as previously described (21, 24). Briefly, the membranes were sequentially incubated with equal volumes of cell culture medium (200 µl), primary biotin-conjugated antibodies, and horseradish peroxidase-conjugated streptavidin. After exposing to X-ray film, the relative signal intensity was determined using the Scion Image software.

Statistical analysis

Data are expressed as mean ± SEM of two independent experiments in triplicates. One-way analyses of variance (ANOVA) followed by the Tukey's test for multiple comparisons were used to compare between different groups. A *P* value less than 0.05 was considered statistically significant.

Results and Discussion

Dermcidin is expressed in sweat glands, and in the absence of an inflammatory stimulus, is constitutively secreted as a full-length protein (1). This full length precursor can be further processed by unknown proteases in human sweat, to form several shorter peptides that exhibit anti-oxidant and antimicrobial activities (Fig. 1A). For instance, the N-terminal

peptide (residue 20–62) has been shown to protect various types of cells against oxidative or hypoxic stresses (25–27). On the other hand, many C-terminal peptides exhibit anti-microbial properties against *S. aureus*, *E. coli*, *E. faecalis*, and *C. albicans* (1). In addition to sweat glands, innate immune cells (e.g., monocytes) also express dermcidin in response to viral infection (28). Furthermore, the full-length dermcidin precursor (residue 22–110) also exhibits bacterial killing activities towards *S. aureus*, *E. coli*, and *P. acnes* (29). Although a C-terminal peptide, DCD-1L, has been shown to activate keratinocytes to produce cytokines (e.g., TNF) and chemokines (e.g., IL-8/CXCL8, CXCL10, and CCL20) (30), it was previously unknown whether the full-length dermcidin precursor exhibits immune modulating properties. Here we provided the first evidence that dermcidin precursor divergently modulated PAMP- and DAMP-induced production of TNF, NO, and chemokines by innate immune cells.

We generated recombinant human dermcidin protein in *E. coli*, and purified it to homogeneity in the absence or presence of a reducing agent (DTT, Figure 1B). Although migrating at a slightly lower rate than the predicted molecular weight, the identity of this recombinant dermcidin was confirmed by Western blotting analysis using a commercially available antibody (Figure 1C). This is consistent with a recent report that recombinant histidine tag-DCD migrated as a 15–16 kDa band on SDS-PAGE gel, even though its molecular weight was determined to be ~9.25 kDa by mass spectrometry (29). Using purified dermcidin, we then tested its immune-modulating properties using macrophage and monocyte cultures. In response to PAMPs (e.g., bacterial endotoxin, LPS) or endogenous cytokines (e.g., CIRP), macrophages released large amounts of nitric oxide (NO, Figure 2). However, dermcidin (DCD) dose-dependently and significantly attenuated both LPS- and CIRP-induced NO release (Figure 2). Although activated monocytes cannot produce NO, they do produce various proinflammatory cytokines or chemokines. We thus stimulated human monocytes with PAMPs (e.g., LPS) or DAMPs (e.g., HMGB1), in the absence or presence of DCD, and measured the relative levels of various cytokines/chemokines using Cytokine Antibody Arrays (Figure 3). As shown in Figure 3, both LPS and HMGB1 elevated the relative levels of several chemokines such as GRO- α and MCP-3. Similarly, dermcidin effectively inhibited LPS- and HMGB1-induced release of GRO- α and MCP-3 from human monocyte cultures (Figure 3). Despite the inhibitory effects on the above chemokines, dermcidin slightly stimulated TNF secretion, an early proinflammatory cytokine that propagates protective innate immune response against microbial infection. In agreement with the stability of dermcidin's anti-bacterial properties over a broad pH range and salt concentrations (1), we found that dermcidin's immune-modulating properties were also relatively stable. Although dermcidin tended to form dimers in the absence of reducing agents (Fig. 1B), its immune modulating properties remained unaltered (data not shown), indicating the relative stability of dermcidin's biological activities *in vitro*.

In animal models of peritoneal microbial infection induced by surgical perforation of the cecum, a technique known as cecal ligation and puncture (CLP) (31), neutralizing antibodies against TNF worsens the outcome (32), supporting a beneficial role of TNF in the innate immunity against bacterial infection. Although appropriate inflammatory responses might be needed for the innate immunity against microbial infection, excessive recruitment of

leukocyte to infection or injury sites might be harmful to the host. As a critical element of the innate immune response, leukocyte recruitment is governed by chemotactic functions of bacterial products and chemokines (such as GRO- α and MCP-3) (33). The dermcidin-mediated suppression of both PAMP- and DAMP-induced chemokines (such as GRO- α and MCP-3) might attenuate leukocyte recruitment to the infection and injury site, and likely prevent excessive inflammatory responses to infection or injury.

Although many anti-inflammatory agents have failed to improve outcomes of many inflammatory diseases (such as sepsis), the investigation of pathogenic cytokines in animal models of diseases has led to the development of successful cytokine-targeting therapeutic strategies (e.g., anti-TNF antibody, infliximab) for autoimmune diseases such as rheumatoid arthritis (34, 35). The dual anti-bacterial (29) and anti-inflammatory (Fig. 2 and Fig. 3) properties may distinguish dermcidin from previously tested anti-inflammatory agents, positioning it as a unique experimental agent for preclinical testing using various animal models of inflammatory diseases. Given the complex and redundant roles of various cytokines and chemokines in various inflammatory diseases, it is now particularly important to test the hypothesis that dermcidin may occupy an important role in the regulation of local or systemic inflammation in preclinical animal models. For instance, injection of bacterial endotoxin directly into the skin (e.g., footpad, subcutaneously) provides a murine model of local inflammation and edema. It is interesting to determine whether local co-administration of dermcidin attenuates paw edema at various time points after endotoxin challenge. Additionally, systemic inflammation can be induced in animals by infusion of bacterial endotoxin such as LPS (31), or surgical perforation of the cecum, a technique aforementioned as CLP (31). It will be important to test whether systemic administration (intraperitoneally or intravenously) of dermcidin would confer a dose-dependent protection against lethal endotoxemia or CLP-induced bacteremia. Future test of this understudied hypothesis may improve our understanding of sweat gland-derived antimicrobial peptides in innate immune regulation, and pave the road for the development of possible therapies for inflammatory diseases.

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Abbreviations used in this paper

CIRP	cold-inducible RNA-binding protein
DAMP	damage-associated molecular pattern molecule
HMGB1	high mobility group box 1
LPS	lipopolysaccharide
NO	nitric oxide
PAMP	pathogen-associated molecular pattern molecule

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A Dermcidin precursor (a.a. 20-110) and proteolytic peptides.

20		63		110
YDPEAASAPGSGNPCHEASAAQKENAGEDPGLARQAPKPRKQRSSLLEKGLDGAKKAVGGLGKLGKDAVEDLESVGKGAVHDVKDVLDSVL				
DCD-1L	(63-110)		SSLLEKGLDGAKKAVGGLGKLGKDAVEDLESVGKGAVHDVKDVLDSVL	
DCD-1	(63-109)		SSLLEKGLDGAKKAVGGLGKLGKDAVEDLESVGKGAVHDVKDVLDSV	
SSL25	(63-87)		SSLLEKGLDGAKKAVGGLGKLGKDA	

Survival (anti-oxidant)

Anti-microbial

B SDS-PAGE
(Coomassie blue)

C Western blot
(Abcam 175519)

Figure 1. Expression and purification of recombinant dermcidin
A). Amino acid sequence of dermcidin precursor and various proteolytic peptides. DCD-1L is a 48 amino acid peptide corresponding to the C-terminal of the full length of dermcidin precursor. **B). Expression and purification of recombinant histidine-tag dermcidin precursor (20–110) (DCD).** Recombinant dermcidin corresponding to residue 20–110 amino acid with an N-terminal histidine tag was expressed in *E. coli* BL21 (DE3) pLysS cells (**Panel B, left gel**), and purified by histidine-affinity chromatography (**Panel B, right gel**) and Triton X-114 extraction to remove contaminating endotoxins. Note that recombinant dermcidin migrated on SDS-PAGE gel as a 12–14 kDa monomer (DCD M) in the presence of a reducing agent (DTT), but migrated as both a monomer and 24–28 kDa dimer (24–28 kDa) in the absence of DTT, suggesting possible cross-linking between dimers

through disulfide bonds. **C). Confirmation of the identify of recombinant protein by Western blotting analysis using dermcidin-specific antibodies.**

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Figure 2. Dermcidin dose-dependently attenuated LPS- and CIRP-induced NO release by murine macrophages

Murine macrophages were stimulated with LPS or CIRP alone or in the presence of recombinant dermcidin (DCD) for 16 hours, and extracellular levels of nitric oxide (NO) were determined by the Griess Reagent. *, $P < 0.05$ versus “– control”; #, $P < 0.05$ versus “+ LPS”, or “+ CIRP” alone.

Figure 3. Dermcidin modulated LPS- and HMGB1-induced chemokine release by human monocytes

Human peripheral mononuclear cells (huPBMCs) were stimulated with LPS (0.8 µg/ml) or HMGB1 (4.0 µg/ml) alone, or in the presence of DCD (1.0 µg/ml) for 16 hours, and extracellular levels of cytokines and chemokines were determined by Cytokine Antibody Arrays (**Panel A, B**). **A, Representative cytokine antibody arrays.** The name of the cytokines and positive controls (“Pos”, or “+Ctrl”) were labeled in the table below. **B, Relative cytokine levels.** The relative cytokine levels were estimated by measuring the

intensity of corresponding signal, and expressed as mean $\pm$ SEM [% of positive controls (“+Ctrl”) of respective arrays] of two independent experiments. *, $P < 0.05$ versus “untreated”; #, $P < 0.05$ versus “+ LPS” or “+HMGB1” alone.

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