EBioMedicine xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

Liposomal Therapy Attenuates Dermonecrosis Induced by Community-Associated Methicillin-Resistant *Staphylococcus aureus* by Targeting α-Type Phenol-Soluble Modulins and α-Hemolysin

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ARTICLE INFO

Article history:
Received 24 April 2018
Received in revised form 5 June 2018
Accepted 12 June 2018
Available online xxxx

Keywords:
Liposomes
Anti-toxin therapy
CA-MRSA
USA300
Skin and soft tissue infections
Phenol-soluble modulins
α-Hemolysin
Dermonecrosis

ABSTRACT

Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA), typified by the pulse-field type USA300, is an emerging endemic pathogen that is spreading rapidly among healthy people. CA-MRSA causes skin and soft tissue infections, life-threatening necrotizing pneumonia and sepsis, and is remarkably resistant to many antibiotics. Here we show that engineered liposomes composed of naturally occurring sphingomyelin were able to sequester cytolytic toxins secreted by USA300 and prevent necrosis of human erythrocytes, peripheral blood mononuclear cells and bronchial epithelial cells. Mass spectrometric analysis revealed the capture by liposomes of phenol-soluble modulins, α-hemolysin and other toxins. Sphingomyelin liposomes prevented hemolysis induced by pure phenol-soluble modulin-α3, one of the main cytolytic components in the USA300 secretome. In contrast, sphingomyelin liposomes harboring a high cholesterol content (66 mol/%) were unable to protect human cells from phenol-soluble modulin-α3-induced lysis, however these liposomes efficiently sequestered the potent staphylococcal toxin α-hemolysin. In a murine cutaneous abscess model, a single dose of either type of liposomes was sufficient to significantly decrease tissue dermonecrosis. Our results provide further insights into the promising potential of tailored liposomal therapy in the battle against infectious diseases.

1. Introduction

Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is an emerging public health threat, especially the predominant, most aggressive USA300 type [1]. Resistant to many conventional antibiotics, it rapidly spreads outside the health care setting and infects even young, healthy individuals with no previous hospital exposure [2]. Infections caused by USA300 mainly manifest as purulent skin and soft tissue infections (SSTIs) [1, 2] or life-threatening diseases such as necrotizing pneumonia and sepsis [1, 2]. CA-MRSA expresses an arsenal of cytotoxic virulence factors influencing the complex interplay between the pathogen and the host's immune system [3–5].

A major virulence-associated feature of *S. aureus* is its capacity to kill host cells, mediated by secreted toxins such as α-hemolysin, leukocidins and phenol-soluble modulins (PSMs) [5]. α-Hemolysin and PSMs have receptor-independent, cytolytic activities if present at high levels [5, 6]. When expressed at lower concentrations, these toxins exert additional pro-inflammatory and/or cytotoxic properties by binding to specific receptors [7–9].

For example, α-hemolysin, plays an important role during staphylococcal pathogenesis, *e.g.* in SSTIs [10–12], mouse lung infection models [13, 14], and sepsis [15]. It is secreted as soluble monomers, binds to and activates the host cell receptor, A Disintegrin And Metalloprotease 10 (ADAM10) [9]. ADAM10 is present on human epithelial, endothelial and myeloid cells but is absent on human erythrocytes [16]. Receptor binding elicits powerful host inflammatory responses and the formation of pore structures in the plasma membrane of host cells that ultimately lead to the destruction of membrane integrity [16, 17].

PSMs are small (2–5 kDa), α-helical, amphipathic peptides exhibiting multiple functions in *Staphylococcus* pathogenesis. α-Type PSMs (PSM-α1, PSM-α2, PSM-α3, PSM-α4, δ-toxin) are ~20–30 amino acids and more cytolytic than the ~45 amino acid β-types (PSM-β1, PSM-β2). PSM-α3 possesses the most potent killing activity among all PSMs [6]. A receptor-independent mode of membrane insertion enables PSMs to kill multiple eukaryotic cell types, including endothelial, epithelial cells and myeloid cells (*e.g.*, erythrocytes, monocytes, and neutrophils) [4, 6]. PSMs also induce receptor-dependent host inflammatory responses [8, 18, 19] and exhibit synergy with other

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https://doi.org/10.1016/j.ebiom.2018.06.016

staphylococcal toxins such as α-hemolysin and the Panton-Valentin leukocidin [PVL [11, 20]], boosting their pathogenic impact. Furthermore, PSMs are involved in biofilm formation, a stress-induced bacterial life-style that confers heightened adaptive antimicrobial resistance [4, 21]. The expression of the *psmα* and *psmβ* genes is strongly enhanced at high bacterial cell densities [6, 22]. MRSA isolates from SSTIs, especially USA300, produce significantly higher levels of PSMs than other *S. aureus* stains [23].

Since the above-mentioned toxins harm host cells and contribute to pathogenicity, sequestration and neutralization of one or more of these virulence factors represents a promising therapeutic approach for attenuating disease severity. Recently, we reported that a liposomal-based toxin-sequestration therapy protected host cells and attenuated bacterial virulence *in vitro* and *in vivo* [24]. Engineered liposomes composed of cholesterol and sphingomyelin (Ch:Sm, 66 mol/% cholesterol) efficiently scavenged a plethora of virulence factors, including cholesterol-dependent cytolysins, phospholipase C and staphylococcal α-hemolysin by mimicking plasma membrane lipid raft-like microdomains that are the preferred target sites for many bacterial toxins [24, 25]. As a single therapy, Ch:Sm liposomes provided only partial protection against staphylococcal and pneumococcal supernatants *in vitro* and in mouse infection models. However their combination with sphingomyelin-only (Sm) liposomes was fully protective, indicating that Sm liposomes neutralized as-yet unidentified virulence factors distinct from those neutralized by Ch:Sm liposomes [24]. The mixture of both liposome types under the trade name CAL02 is currently being tested in a clinical trial against severe pneumococcal pneumonia (ClinicalTrials.gov Identifier: NCT02583373).

Here we demonstrate that Sm, but not Ch:Sm liposomes bound and neutralized hemolytic virulence factors present in USA300 supernatants and protected human red blood cells (RBCs), peripheral blood mononuclear cells (PBMCs) and bronchial epithelial 16HBE140- (HBE) cells from rapid cell lysis. Mass spectrometric analysis of bacterial proteins bound by the Sm liposomes revealed α-type PSMs as an interacting target. Sm liposomes, but not Ch:Sm liposomes decreased hemolysis induced by purified recombinant PSM-α3. In contrast to Sm liposomes, Ch:Sm liposomes efficiently bound α-hemolysin. Furthermore, both types of liposomes attenuated CA-MRSA virulence by significantly reducing dermonecrosis in a murine cutaneous abscess model.

2. Materials and Methods

2.1. Liposomes

Unilamellar cholesterol:sphingomyelin (Ch:Sm, 66 mol/% cholesterol, 40 mg/ml, diameter 130 nm) and sphingomyelin (Sm, 40 mg/ml, diameter 60 nm) liposomes in sodium Tyrode's buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES; pH = 7.4) were provided by Lascco (Geneva, Switzerland, product name CAL02).

2.2. Bacterial strains and supernatants

The MRSA USA300 pulse field type isolate LAC (USA300) was kindly provided by Michael Otto (National Institute of Health, Bethesda, MD) and bioluminescent LAC USA300 was kindly provided by Scott Stibitz (Food and Drug Administration, Silver Spring, MD). Bacteria were cultured in Tryptic Soy Broth (TSB, Becton Dickinson). Overnight cultures were diluted in fresh TSB to an optical density OD_{600} of 0.1 and incubated at 37 °C under shaking conditions for 22 h. Bacteria were pelleted (5000 ×*g*, 10 min) and the resulting supernatants were filter sterilized (pore size 0.2 μm, Nalgene).

If indicated, bacterial supernatants were high-speed centrifuged (100,000 ×*g*) at 4 °C for 1 h. The resulting supernatants were treated with liposomes or sodium Tyrode's buffer (vehicle) for 5 min. Subsequently, liposomes were pelleted (100,000 ×*g*) at 4 °C for 1 h. The resulting liposome-free supernatants were used for the cytotoxicity assay in Fig. 1e. The liposome/toxin or vehicle/toxin pellets were applied to SDS-PAGE or mass spectrometric analysis.

2.3. Human cells

Peripheral blood mononuclear cells (PBMCs) and red blood cells (RBCs) were isolated from the blood of healthy, consenting human volunteers (following the University of British Columbia ethics guidelines). Blood was collected in sodium heparin anticoagulant collection tubes (BD Biosciences), diluted in phosphate buffered saline (PBS, ThermoFisher/Gibco) and layered onto Lymphoprep density gradient medium (STEMCELL Technologies). After centrifugation (500 ×*g* for 20 min) the buffy coat was transferred to a new tube, washed three times with PBS and resuspended in RPMI-1640 Medium (+25 mM HEPES, +L-Glutamine, GE Healthcare) supplemented with 10% fetal bovine serum (FBS, ThermoFisher/Gibco). PBMCs were seeded at density of 100,000 cells and rested.

RBCs were collected from the bottom of the density gradient, washed three times with PBS and stored for a maximum of 4 weeks in Alsever's solution (Sigma Aldrich).

The human bronchial epithelial cell line 16HBE14o- (HBE, RRID: CVCL_0112) was kindly provided by Dr. D. Gruenert (University of California San Francisco). HBE cells were maintained in MEM medium (ThermoFisher/Gibco) supplemented with 10% FBS, 2 mM L-glutamine (ThermoFisher/Gibco) and 1% penicillin/streptomycin (ThermoFisher/Gibco) at 37 °C in 5% CO_2. Cells were dissociated with 0.25% trypsin-EDTA (ThermoFisher/Gibco) at 80–90% confluency.

2.4. Hemolysis assay

Prior to use, RBCs were washed three times with TSB (1000 × g, 10 min). Serial dilutions of liposomes were incubated with bacterial supernatants (50 μl) and 2% RBCs in a 200 μl reaction volume with TSB in microtiter plates (Falcon). The hemolytic activity of purified staphylococcal PSM-α3 peptide (IBT Bioservices, Cat# 1401–004) was assessed in PBS. Triton X-100 (2% v/v, Sigma–Aldrich)-treated RBCs served as a positive control and TSB-treated RBCs as a negative control. After incubation for 1 h at 37 °C, RBCs were pelleted (1000 × g, 10 min) and the hemoglobin content in the supernatant was measured at OD_{450} (reference 630 nm) using a microplate reader. Relative hemolysis (%) was calculated as $(\Delta OD_{sample} - \Delta OD_{negative\ control})/(\Delta OD_{positive\ control} - \Delta OD_{negative\ control}) \times 100$.

2.5. Cytotoxicity assays

Cytotoxicity assays were performed in cell-culture treated microtiter plates (Costar). 100,000 PBMCs were seeded directly after their isolation in RPMI +10% FBS (100 μl) and rested for 1 h. 40,000 HBE cells were seeded two days prior treatment in MEM + 10% FBS and grown to confluency. Shortly before treatment the medium was replaced with MEM + 1% FBS (100 μl). Bacterial supernatants (12.5 μl PBMCs, 50 μl HBE cells) and liposomes (300 μg/ml) or sodium Tyrode's buffer (vehicle) were added (total reaction volumes: 150 μl PBMCs, 200 μl HBE cells). Triton X-100 (2% v/v, Sigma–Aldrich)-treated PBMCs or HBE cells served as a positive control and TSB-treated PBMCs or HBE cells as a negative control. After incubation for 1 h at 37 °C, PBMCs or HBE cells were centrifuged (500 ×*g*, 5 min) and the lactate dehydrogenase (LDH) content in the supernatant was assessed with the Cytotoxicity Detection Kit^{Plus} (Roche) according to the manufacturer's instructions. Relative LDH release (%) was calculated by $(\Delta OD_{sample} - \Delta OD_{negative\ control})/(\Delta OD_{positive\ control} - \Delta OD_{negative\ control}) \times 100$.

Please cite this article as: Wolfmeier H, et al, Liposomal Therapy Attenuates Dermonecrosis Induced by Community-Associated Methicillin-Resistant *Staphylococcus aureus*..., EBioMedicine (2018), https://doi.org/10.1016/j.ebiom.2018.06.016