Bronchoalveolar Lavage Microvesicles Protect Burn-Injured Mice from Pulmonary Infection

Teresa C Rice, MD, Amanda M Pugh, MD, Brent T Xia, MD, Aaron P Seitz, MD, Brynne E Whitacre, BS, Erich Gulbins, MD, PhD, Charles C Caldwell, PhD

BACKGROUND: *Pseudomonas aeruginosa* is a major cause of morbidity and mortality among burn patients, despite antibiotic therapy. There is a need to identify innate immune defenses that prevent *P aeruginosa* infection in injured adults in an effort to find therapeutic alternatives to antibiotics. Here, we tested our hypothesis that microvesicles (MVs) in bronchoalveolar (BAL) fluid have a role in the immunity of the lung in response to pathogens.

STUDY DESIGN: Microvesicles were isolated from murine BAL fluid, quantified using Nanoparticle Tracking Analysis, and injected into burn-injured mice before *P aeruginosa* infection. Survival was assessed and BAL bacterial loads enumerated. Neutrophil number and interleukin 6 activity were determined. Lungs were harvested and sphingosine (SPH) content analyzed via immunohistochemistry. Antimicrobial effects of MVs and SPH-enriched MVs were assessed in an in vitro assay.

RESULTS: Burn-injured mice have reduced BAL MV number and SPH content compared with sham. When BAL MVs from healthy mice are administered to injured mice, survival and bacterial clearance are improved robustly. We also observed that intranasal administration of MVs restores SPH levels after burn injury, MVs kill bacteria directly, and this bacterial killing is increased when the MVs are supplemented with SPH.

CONCLUSIONS: Using a preclinical model, BAL MVs are reduced after scald injury and BAL MV restoration to injured mice improves survival and bacterial clearance. The antimicrobial mechanisms leading to improved survival include the quantity and SPH content of BAL MVs. (J Am Coll Surg 2017;225:538–547. © 2017 by the American College of Surgeons. Published by Elsevier Inc. All rights reserved.)

Disclosure Information: Nothing to disclose.

Support: Supported by Shriners Hospitals for Children #85000-CIN-17 (CCC), R01 GM100913 (CCC), and T32 GM08478 (TCR AMP) from the National Institute of General Medical Sciences.

Disclaimer: The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health.

Awarded first prize in basic science at the American College of Surgeons Region 5 Resident Trauma Paper Competition and the American College of Surgeons Committee on Trauma Resident Trauma Paper Competition, Washington, DC, March 2017.

Received April 23, 2017; Revised June 23, 2017; Accepted June 23, 2017.

From the Division of Research, Department of Surgery, University of Cincinnati, Cincinnati, OH (Rice, Pugh, Xia, Seitz, Whitacre, Gulbins, Caldwell) and Department of Molecular Biology, University of Duisburg-Essen, Essen, Germany (Gulbins).

Correspondence address: Charles C Caldwell, PhD, Division of Research, Department of Surgery, University of Cincinnati, MSB SRU G479 ML 0558, 231 Albert Sabin Way, Cincinnati, OH 45267-0558. email: charles.caldwell@uc.edu

Pneumonia is the leading cause of mortality from nosocomial infections in patients admitted to ICUs.[1] Mechanically ventilated patients are particularly vulnerable to pneumonia infection. Ventilator-associated pneumonia is thought to be caused by the endotracheal tube bypassing the innate immune defenses of the upper respiratory tract and allowing bacteria into the lung.[2] *Pseudomonas aeruginosa* (PA) is a virulent gram-negative pathogen that has the ability to form a biofilm on the inside of endotracheal tubes, increasing the likelihood of pneumonia developing.[3] It is more prevalent in susceptible patient populations, including immunosuppressed, cystic fibrosis, and burn patients.[4] With improvements in wound care and resuscitation, pneumonia is a major cause of morbidity and mortality in the burn patient population.[1,5,6] In particular, PA is the most frequently isolated bacterial pathogen.[1,7] Further complicating PA treatment is rising antibiotic resistance, which is contributing to

http://dx.doi.org/10.1016/j.jamcollsurg.2017.06.010
ISSN 1072-7515/17

Abbreviations and Acronyms

BAL = bronchoalveolar lavage
CFU = colony-forming unit
CLP = cecal ligation and puncture
IL = interleukin
MP = macrophage
MV = microvesicle
NDMV = neutrophil-derived microvesicle
PA = *Pseudomonas aeruginosa*
PBD1 = post-burn day 1
SPH = sphingosine

higher mortality rates.[3,4,6] Due to the high associated mortality, there is a need to identify innate immune defenses that prevent PA infection in healthy adults and find a therapeutic alternative or complement to antibiotics.

Sphingosine (SPH) is a lipid found in a variety of cell membranes and cell membrane-derived microvesicles (MVs) that has antimicrobial properties.[8] It has been shown to contribute to innate antimicrobial activity of skin and mucosal surfaces.[9-11] In particular, it has been reported to protect skin from bacterial colonization[12] and protect lungs from pulmonary infection in cystic fibrosis[8] and burn injury models.[6] Sphingosine is generated as the product of hydrolysis of sphingomyelin to ceramide via sphingomyelinase and subsequently ceramide to SPH via the enzyme ceramidase.[8,11] The susceptibility to pulmonary infection seems to be related to the lipid composition of the cell membranes, with alterations in the ceramide/sphingosine pathway resulting in altered immunity.[6,8] Ceramide levels have been shown to be increased pathologically, and SPH levels are decreased in tracheal epithelial cells of cystic fibrosis and burn-injured murine models.[6,8] Prophylactic treatment with aerosolized SPH decreased bacterial load and improved survival in these studies.[6,8] However, the role of tracheal epithelial cell-derived MVs, which inherently contain SPH, in the prophylactic treatment of PA pulmonary infection has not been investigated.

Microvesicles are small vesicles derived from cell membranes of heterogeneous density and composition.[13] During cellular activation and apoptosis, MVs are known to transfer information via cell-to-cell communication.[14,15] Although found in healthy individuals, MV quantity and type are altered during inflammatory disease.[16-18] Microvesicle signals can be mediated by a variety of molecules, including protein, lipids, and carbohydrates. These signals depend largely on the parent cell, which can be lymphocytes, platelets, leukocytes, endothelial, and epithelial cells.[14,15] Platelet-derived MVs are the most abundant and neutrophil-derived MVs are the dominant type generated at the site of infection.[14,19,20] Neutrophil-derived MVs have been shown to increase the inflammatory response and increase mortality.[14,19] Although it is known that MVs in bronchoalveolar (BAL) fluid have a role in the immunity of the lung in response to pathogens, the mechanism is unknown.[15]

The role of BAL MVs in response to PA pneumonia in burn patients has not been investigated. Given previous data in the burn-injured murine model, we hypothesize that the quantity of BAL MVs will be reduced after burn injury and treatment of injured mice with BAL MVs from healthy donors will improve survival and bacterial clearance. In addition, we hypothesize that the mechanism leading to improved survival is related to the quantity and SPH content of BAL MVs.

METHODS

Mice

Six-week-old male CF-1 mice were purchased from Charles River Laboratories. They were housed in standard environmental conditions, allowing them to acclimate 1 week before experiments. All experiments were conducted by protocols (#08-09-19-01) approved by the Institution Animal Care and Use Committee of the University of Cincinnati.

Burn injury

Mice were subjected to a full-thickness scald injury as described previously.[21] Briefly, mice were weighed and anesthetized with 4.5% isoflurane in oxygen before clipping hair from their dorsal surface. They were placed in a template according to their weight that exposed 28% total surface body area. They were immersed in 90.0°C water for 9 seconds to produce a full-thickness scald injury to the exposed surface. After scald injury, mice received 1.5 mL sterile normal saline via intraperitoneal injection for resuscitation and allowed to recover on 42°C heating pad for 3 hours. Sham mice received the same treatment except they were immersed in room temperature water.

Microvesicle isolation

Microvesicles were isolated as described previously.[19] In brief, BAL fluid was collected from sham and post-burn day 1 (PBD1) mice and centrifuged at 450 *g* for 10 minutes at room temperature to form a cell pellet. Supernatant was collected and centrifuged at 10,000 *g* for 10 minutes at room temperature to remove any residual cells. Supernatant was then centrifuged at 25,000 *g* for 30 minutes at room temperature to pellet MVs. Microvesicle pellets were resuspended in 1 mL RPMI and 1:100 MV