Mitochondrial Damage Associated Molecular Patterns From Femoral Reamings Activate Neutrophils Through Formyl Peptide Receptors and P44/42 MAP Kinase

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INTRODUCTION
Acute lung injury and adult respiratory distress syndrome (ALI/ARDS) occur after fractures in a sporadic entity often termed “fat embolism syndrome.” Fat embolism syndrome is hard to distinguish from ALI/ARDS occurring after sepsis and may be associated with reamed nailing more than other methods of fixation. Current concepts emphasize that fracture hematomas are rich in inflammatory mediators that can activate immune cells like neutrophils (PMN) that can injure the lung, but it is unknown what the primary events are causing fractures to be rich in mediators. Understanding the events linking mechanical injury to immune organ dysfunction is essential if effective therapies are to be developed.

Bacteria can cause inflammation by releasing lipids (like lipopolysaccharide) or n-formyl peptides. Such “pathogen-associated molecular patterns” activate cells through “pattern-recognition receptors” like toll-like receptor-4 (responds to lipopolysaccharide) or the receptors formyl peptide receptor (FPR)-1 and FPRL-1 that respond to formyl peptides (FP). Human genomic proteins are not formylated, but mitochondria resemble bacteria in many ways, having FP and circular DNA (mitochondrial DNA) with nonmethylated repeats like bacterial symbionts. These observations led to the conclusion that mitochondria were once free-living bacteria that became intracellular symbionts. They also suggest that when mitochondrial molecular patterns are released from cells by injury, they might activate immunity by mimicking bacterial motifs. Intrinsic molecular motifs like this are referred to as “damage associated molecular patterns” (DAMPs) or “alarmins.” We have shown that mitochondrial FPs activate PMN and that mitochondrial DNA is released in shock.

MATERIALS AND METHODS

Research Compliance
Studies were performed under the supervision of the Institutional Review Board of Beth Israel Deaconess Medical Center and Harvard Medical School. Fracture reaming specimens were collected under waiver of consent for discarded materials. Consent was obtained for sampling and archiving of trauma plasma samples from the patients or their legally
authorized representative whenever such consent was available. Animal experimentation was approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

Patients and Biologic Samples

Femoral reamings were collected intraoperatively from 10 patients with diaphyseal femur fractures. Patients were 18 to 54 years old and had isolated closed injuries sustained in motor vehicle traumas. Specimens were kept at 4°C while processed. Specimens were spun to remove gross particulates. The residual cellular material was subjected to a standard mitochondrial isolation protocol using a kit (Pierce, Rockford, IL). Plasma specimens were obtained when consent for blood draw was available \( n = 5 \). Mitochondria were sonicated and spun at 12,000 g. The supernatant was assessed for biologic activity of MTD. Samples of fracture fluid were also spun at 12,000 g and the supernatants assayed by quantitative polymerase chain reaction in triplicate to assay mitochondrial DNA.

Animals

Male Sprague-Dawley rats (250–350 g; Charles River, Wilmington, MA) were acclimatized under barrier sustained conditions (25°C, 12-hour light/dark cycles, water and chow ad libitum). Animals were cannulated as per our published methods\(^{13}\) and injected \( n = 3 / \text{group} \) with MTD from human fracture hematomas or media. Animals were euthanized 1 hour later. Lungs were harvested and frozen at −80°C for later analysis.

Reagents and Chemicals

We purchased fMLP, EGTA, and DMSO from Sigma (St. Louis, MO); Fura2-AM from Molecular Probes (Eugene, OR); antihuman FPR1 from R&D (Minneapolis, MN); Hanks’ balanced salt solution and phosphate-buffered saline from Gibco (Grand Island, NY); antihuman metalloproteinase-9 from Chemicon (Billerica, MA); and phospho-p44/42 MAP kinase (Thr202/Tyr204) and p44/42 MAP kinase antibodies from Cell Signaling (Danvers, MA).

Details of Mitochondrial Isolation

Mitochondria were isolated using a Mitochondria Isolation Kit for tissue (Pierce, Rockford, IL) according to the manufacturer’s dounce-soft tissue protocol under sterile conditions at 4°C and then stored on ice for further processing.

Preparation of Mitochondrial Damage Associated Molecular Patterns

Mitochondria from 200 mg of tissue were suspended in 1 mL of buffer (Hanks’ balanced salt solution for chemotaxis assays and HEPES for calcium assays). Protease inhibitor cocktail (1:100) was added. Suspensions were subjected to sonication on ice (VCX130-Vibra Cell; Sonics and Materials, Newtown, CT) at 100% amplitude three times for 30 seconds each. The disrupted mitochondria were centrifuged at 12,000 rpm for 10 minutes at 4°C. Supernatants were removed and stored at −20°C for experiments. Protein concentration was determined by BCA assay. We have previously noted that there are no interspecies differences in the responses to mitochondria between human and rat. Immune cells from rat and man respond equally to MTD from their own and the other species.\(^{5,8,9}\)

Neutrophil Isolation

Detailed protocols are described elsewhere.\(^{13,14}\) Briefly, heparinized volunteer blood is centrifuged and platelet-rich plasma removed. The buffy coat and 2 cm of red blood cells are layered onto Polymorphoprep gradient (Robbins Scientific, Sunnyvale, CA) and spun (1500 rpm, 30 minutes). PMNs are collected and osmolality restored for 5 minutes with an equal volume of 0.45% NaCl. PMNs are washed, centrifuged, and a hypotonic lysis is done on ice to remove residual red blood cells. The preparation contains 98% or more neutrophils (cytospin) that are 99% or greater viable (Trypan blue). PMN pellets are resuspended in Hanks’ balanced salt solution with 5% fetal bovine serum for chemotaxis assays or in HEPES buffer with 0.1% bovine serum albumin for calcium and oxidative burst experiments.

Calcium Dye Loading

PMNs were incubated in 2 μM fura-2AM (30 minutes, 37°C). Cells were divided into 200-μL aliquots and kept on ice. Aliquots were rewarmed to 37°C before experiments, centrifuged, resuspended in 200 μL of HEPES, and loaded into a cuvette containing 2.8 mL of the same buffer. Experiments were begun in “calcium-free” media (0.3 mM EGTA added) and 1.8 mM CaCl\(_2\) was added to the media as indicated.

Spectrofluorometry

PMN [Ca\(^{2+}\)], was measured using fura-2AM in a spectrofluorometer (Fluoromax-2; Jobin-Spex, Edison, NJ) using our modifications of the methods of Grynkiewicz et al.\(^{13}\) Fracture fluids were added to the media at up to 20%. Higher concentrations of admixed protein solutions cause unacceptable autofluorescence.\(^{14}\)

Western Blots

Rat lung homogenates, human PMN supernatants, and lysates were boiled for 5 minutes in sodium dodecyl sulfate sample buffer. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis using 4% to 20% Tris-glycine polyacrylamide gradient gels (Novex, San Diego, CA). Separated proteins were transferred to nitrocellulose (0.45-μm pore size; Immobilon-P; Millipore, Bedford, MA). Matrix metalloproteinase-9, Phospho-p44/42, and p44/42 MAPK were immunoblotted with specific antibodies and detected using enhanced chemiluminescence (Amersham, GE, Buckinghamshire, UK). Membranes were stripped and reprobed with β-actin (Santa Cruz, CA) to assess loading. Densitometry was performed with the Scion Image program, Scion Corporation, Frederick, MD.

Quantitative Polymerase Chain Reaction

DNA was isolated from 200 μL of volunteer plasma as controls. DNA was isolated from the same volume of reaming fluid of patients with femur fractures or their plasma when available. DNA isolation was performed using QIAamp DNA Mini Kit (Qiagen, Valencia, CA.). DNA concentration and quality was measured with a Nanodrop spectrophotometer.
A total of 7.5 μL of DNA was used for each polymerase chain reaction. SYBR green polymerase chain reaction was performed on volunteer plasma, patient plasma, and fracture fluid using primers targeting cytochrome B (CytB) that were specific (on “BLAST” analysis) for mitochondrial DNA. Primers (1R: CGAAGTTTCATCATGCAGG and 1F: ATGACCCCAATACCGAAAAAT) were synthesized by Invitrogen, Carlsbad, CA.

Statistical Analyses

All [Ca\(^{2+}\)] transient results reported are measured as the mean change from basal [Ca\(^{2+}\)], in nanomoles per liter (nM). Quantitative polymerase chain reaction was assessed as cycle count number (Ct): increasing cycle number to detection reflects decreasing abundance of the target. Data were assessed for significance using Student’s (unpaired) t test or analysis of variance where appropriate. Post hoc analyses for analysis of variance were selected by the SigmaStat program (Systat Software, Richmond, CA). Data are reported as the mean ± standard error of mean and statistical significance is accepted at a P value < 0.05. All examples of molecular studies shown are representative of three or more replications.

RESULTS

Mitochondrial Damage Associated Molecular Patterns in Fracture Patients

Mitochondrial DNA is a biomarker for the presence of MTD. We found that mitochondrial DNA was close to undetectable in volunteer plasma (Fig. 1). Fracture fluids contained approximately twofold (greater than 100,000-fold) more mitochondrial DNA than volunteer plasma. Circulating plasma from patients with fractures had 500- to 1000-fold more mitochondrial DNA than volunteer plasma.

Mitochondrial Damage Associated Molecular Patterns Activate Polymorphonuclear [Ca\(^{2+}\)] Flux

PMNs were stimulated with fracture supernatants at 10% and 20% concentration. Experiments were performed in low-Ca\(^{2+}\) environment followed by recalcification of the medium thus visualizing first intracellular Ca\(^{2+}\) flux and then Ca\(^{2+}\) entry into the cell (Fig. 2). Fracture supernatants caused immediate (t = 30 seconds) Ca\(^{2+}\) release by endosomal stores followed by enhanced entry of Ca\(^{2+}\) into the cell (t = 150 seconds).

Mitochondrial Damage Associated Molecular Patterns Activate Polymorphonuclear P44/42 MAP Kinases

PMNs activated by mitochondria from femur fractures (FFx) (10 μg protein/mL, 10 minutes) were assessed for phosphorylation of p44/42 mitogen-associated protein kinase (MAPK). Total p44/42 and β-actin were used as internal standards. MTD cause brisk phosphorylation of p44/42-MAPK (Fig. 3). Stimulation after treatment with cyclosporine H (CsH, 1 μM, a specific inhibitor of FPR1) completely inhibited activation. Thus, FFx activate PMN P44/42 MAPK through formyl peptide receptors.

Mitochondrial Damage Associated Molecular Patterns Cause Polymorphonuclear to Release Matrix Metalloproteinase-9

Excessive matrix metalloproteinase release in inflammation contributes to bystander organ injury through many pathways. We found PMN released matrix
metalloproteinase-9 in a dose-dependent fashion when stimulated by MTD from femoral reamings (Fig. 4). Like with p44/42-MAPK, the effect was completely reversed by CyH. Matrix metalloproteinase-9 release was completely blocked by anti-FPR1 antibodies. These findings show PMN degranulation of matrix metalloproteinases is activated by MTD in FFx through formyl peptide receptors, likely FPR1 (n = 3 replications/condition, *P < 0.05 by analysis of variance/Tukey’s test).

**Femur Fracture Mitochondrial Damage Associated Molecular Patterns Cause Polymorphonuclear Interleukin-8 Release**

Activated PMN release interleukin-8, which activates PMN and recruits further PMN. Both interleukin-8 and responses to it are increased in ALI/ARDS. Here, PMNs were induced to produce interleukin-8 by exposure to MTD from femoral reamings (Fig. 5). The dose response suggests this may occur at the concentrations found in circulating plasma during reaming.

**Pulmonary Inflammation in Response to Fracture Mitochondrial Damage Associated Molecular Patterns**

MTD from human femoral reaming was injected into rats intravenously. Rats were euthanized 1 hour later. Whole lung homogenates were prepared and assayed for phosphorylation of p44/42 (Fig. 6). We found brisk phosphorylation of p44/42, suggesting FFx MTD cause ingress of activated inflammatory cells into the lung.

**FIGURE 3.** Neutrophils were exposed to mitochondrial damage associated molecular patterns (MTD) derived from femur fractures (FFx) (MTD) and assayed by Western blot for the phosphorylation of p44/42 mitogen-associated protein kinase. Damage associated molecular patterns (DAMPs) from FFx rapidly activated this key polymorphonuclear (PMN) kinase. Activation was strongly inhibited by cyclosporine H, an inhibitor of the formyl peptide receptor-1 (FPR1). Total p44/42 and β-actin are shown as controls. *P < 0.05 (analysis of variance/Tukey’s test).

**FIGURE 4.** Polymorphonuclear (PMN) release matrix metalloproteinase-9 (MMP-9) after exposure to femur fracture (FFx) mitochondrial damage associated molecular patterns (MTD). PMN were exposed to FFx MTD (10 minutes) at the concentrations noted. Supernatants were assayed for MMP-9 by Western blot. MTD caused brisk degranulation of MMP-9 (*P < 0.05; analysis of variance/Tukey test). Release was inhibited by cyclosporine H, an inhibitor of formyl peptide receptor-1 (FPR1), or by monoclonal antibodies to FPR1 (αFPR1).

**FIGURE 5.** Polymorphonuclear (PMN) produce interleukin-8 (IL-8) after exposure to mitochondrial damage associated molecular patterns (MTD) from femur fracture (FFx) reamings. Ten percent and 20% MTD caused brisk release of IL-8 at 4 hours (analysis of variance P < 0.01; post hoc Holm/Sidak P < 0.05).
FIGURE 6. Mitochondrial damage associated molecular patterns (MTD) from femur fracture (FFx) reamings were injected into rats intravenously. One hour later, rats were euthanized and whole lung homogenates were assayed for activation of p44/42 by Western blot. We found brisk phosphorylation of p44/42, suggesting the onset of pulmonary inflammation. *P = 0.002 versus naive (analysis of variance/Holm-Sidak).

DISCUSSION

Activation of innate immune phagocytic function is required for the clearance of injured tissue that must precede wound repair. What we see here is that the same primary mediators initiating local innate immunity can also precipitate systemic activation of innate immunity, clinically manifest as systemic inflammatory response syndrome. We believe systemic inflammatory response syndrome is universal after major fracture/soft tissue injury but that its intensity varies widely. This may reflect factors related to the local wound or reflect variable host response. However, in any case, fracture/soft tissue injury and its management can contribute to clinical systemic inflammatory response syndrome indistinguishable from sepsis without an identifiable infection. Moreover, these manifestations can include ALI or ARDS.

Here, we show femoral fracture wounds (and their repair by reamed nailing) can release mitochondrial debris into the wound and into the systemic circulation. Because mitochondria are evolutionarily derived from bacteria,10,11 we hypothesized their release by cellular disruption would expose the host to immunologically active “danger signals.” Our findings demonstrate that femur fractures and reamed repairs do indeed release mitochondrial DAMPs and that these FFx-derived DAMPs are capable of activating innate immune cells and causing pulmonary inflammation in an animal model.

Our findings support the novel paradigm that fracture/soft tissue injury contribute to the genesis of clinical SIRS by local release of intracellular, mitochondrial-derived DAMPs. Our current studies are limited to evaluation of PMN activation and biochemical studies performed in rats treated with MTD. Moreover, other intracellular motifs can act as “alarmins”16,18 and such molecules are also likely to participate in local wound repair as well as systemic inflammation. Thus, biologic response modifications based on the effects of MTD alone would be premature and must be approached with caution.

We therefore hypothesize that release of MTD from injured tissues forms a link between tissue trauma and sterile SIRS that can predispose to inflammatory lung injury. More research is required to determine the extent to which release of mitochondrial products by mechanical disruption or other forms of tissue injury is responsible for systemic PMN activation and the evolution of SIRS after clinical injury.

REFERENCES